Structural Investigations on the Water-soluble Polysaccharides of Green Algae

A Thesis

Presented for the Degree of Doctor of Philosophy

by

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November 1958

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ALGAL POLYSACCHARIDES

It is possible to divide polysaccharides in general into two groups: structural polysaccharides are often insoluble and form the skeletal structure of plants, while reserve polysaccharides may be broken down into simpler units by the action of enzymes present in the organism. Such differentiation is to be observed among the polysaccharides of marine algae; structural materials are exemplified by alginic acid and the sulphated galactans, while reserve food is stored in the form of laminarin and Floridean starch.

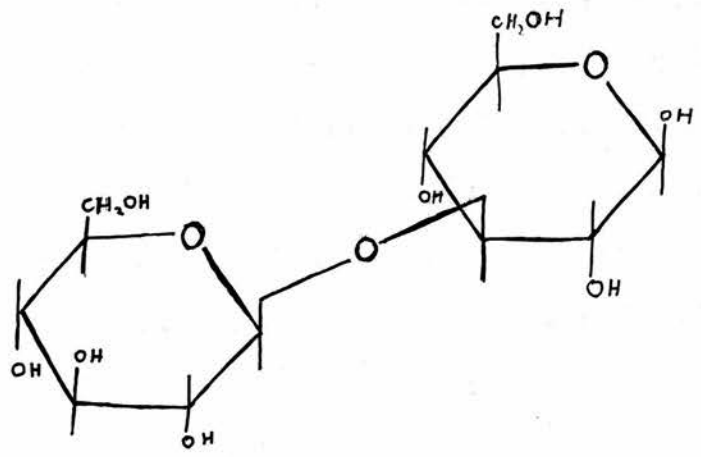
It has been estimated that more than 80% of the dry weight of some seaweeds consists of carbohydrate material, and so it is not surprising that chemical investigation of these plants has tended to centre on their sugar constituents (1). In general, polysaccharides of the red and brown algae (Rhodo- and Phaeophyceae) have been investigated in more detail than those of the green and blue-green varieties (Chloro- and Cyanophyceae). Structural experiments on these polysaccharides have usually followed the normal procedures of hydrolysis, partial hydrolysis, oxidation, and investigation of the methylated hydrolysate. Algal polysaccharides may be considered under several headings such as glucans and xylans, according to their basic monosaccharide repeating unit.

Glucans (a) Laminarin

Laminarin, the main reserve polysaccharide of the brown algae, is easily extracted by dilute

acid from the fronds of Laminaria sp. (2). Two varieties of the polysaccharide, which differ markedly in their solubility in water, have been distinguished (3). The amount of laminarin present in the brown seaweeds varies with the season, being high in the autumn, and low in the spring (4).

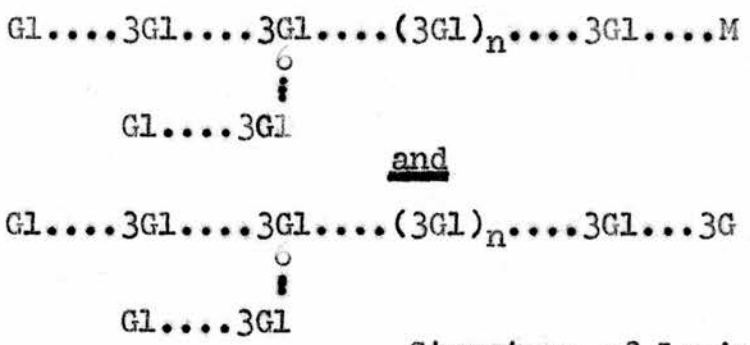
Hydrolysis confirmed that more than 90% of the laminarin molecule consists of D-glucose units. Methylation and hydrolysis afforded a large preponderance of 2:4:6-tri-O-methylglucose, indicating that the molecule consists mainly of 1:3-linked glucose residues (5). The isolation and characterisation of the disaccharide, laminaribiose (3-O-β-(D-glucopyranosyl)-D-glucose), is consistent with this structure (6). Such a polysaccharide, having no 1,2-glycol groupings in the basic repeating unit, is largely immune to oxidative attack by the periodate ion (cf. Part II, this thesis)(7).



Laminaribiose

It has been shown that mannitol occurs at the reducing end of some of the laminarin chains (8). Recent isolation of ca. 1% of oligosaccharides containing β-1:6

links, indicates a slight degree of branching in the molecule (9). It is considered that the main difference between the two varieties of laminarin lies in the greater proportion of mannitol and β-1:6 links in the more soluble material.



Structure of Laminarin

Chain-length determinations on laminarin, based on the amount of tetra-O-methylglucose obtained from methylated hydrolysates, have led to the figure of twenty sugar units per chain; ultra-centrifuge experiments have given a value in excess of sixty units per molecule, again indicating some degree of branching in the polysaccharide (10). Estimations of reducing power, together with analysis of the amount of mannitol present, have given values of nineteen and twenty-four as the chain-length of soluble and insoluble laminarin respectively (11).

(b) Floridean Starch

The presence of an iodine-staining polysaccharide in the aqueous extracts of red weeds was first reported by Colin (12). The high conversion to glucose (96%), together with high specific rotation ($[\alpha]_D$ + 150°) and reaction with iodine, pointed to the obvious similarity with glucans of

the amylopectin type. X-ray analyses have shown that the molecules are physically similar to those of potato starch (13). There is a possibility that anomalous 1:3-links are present in the Floridean starch molecule in slight proportion; the isolation, from a partial hydrolysate, of ca. 0.5% of nigerose (3-O-α-(D-glucosyl)-D-glucose)(14),is in keeping with the occurrence of a small amount of glucose in the hydrolysate of the oxopolysaccharide (15).

(c) Algal Cellulose

In common with land plants, although generally not in as great amounts, many seaweeds contain cellulose in at least the inner cell walls of the plant. Such algal cellulose was first reported by Stanford as the insoluble residues remaining after extraction of alginic acid from brown seaweeds (16). These residues, after purification by reprecipitation from cuprammonium solution, had all the chemical properties of a degraded form of cellulose (17). More recently, cellulose I has been identified among the residues left after extraction of the green weeds Cladophora rupestris and Chaetomorpha melagonium (18). On the other hand, the cell-walls of Ulva and Enteromorpha sp. give X-ray diagrams basically different from that of cellulose I.

Algal Xylan

D-Xylose has been found as a product of hydrolysis of several green and red seaweed extracts. Dilute acid extraction of the red weed, /

Rhodymenia palmata, affords 1% yield of pure xylan (19). Methylation results, confirmed by periodate oxidation data, indicate that the molecule is composed of 1:3- and 1:4-linked xylopyranose units in the proportion of 1 to 4. Since prolonged attempts at fractionation failed to give any indication of heterogeneity, it must be concluded that both linkages are present in the same molecule. More recently, a pure xylan has been isolated from the green weed, Caulerpa filiformis, and proved to consist entirely of 1:3-linked residues (20).

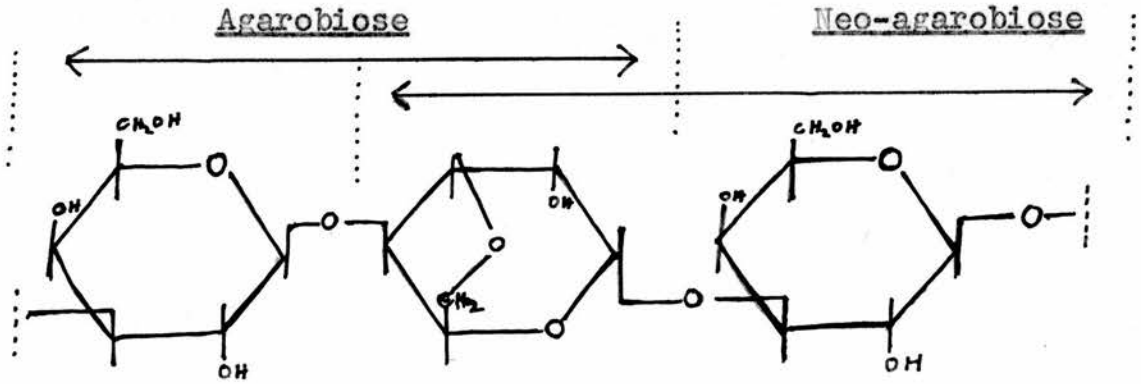
Galactans : (a) Agar

Both D- and L-galactose occur very widely in polymeric form among the polysaccharides of the Rhodophyceae. Their occurrence is often associated with the presence of esterified sulphate groupings, first reported by Haas (21). The nature of this grouping, together with its resistance to normal methods of investigation, has led to much difficulty in the investigation of these polysaccharides.

Agar is usually obtained by water extraction of red weeds of the Florideae class. It is likely that commercial samples vary somewhat in their chemical and physical constitution; as normally available, the sulphate content of different samples ranges between 0.3 and 1.4%, corresponding to one ester grouping per 200 - 40 sugar units (22). Hydrolysis of methylated agar affords 2:4:6-tri-O-methylgalactose in 65% yield; in conjunction with the low specific rotation of the polysaccharide, it was thus indicated that the main feature of the molecule was a chain of 1:3-linked β-D-galactopyranosyl units (23).

From a mixture obtained by remethylating the hydrolysis products of methylated agar, a second sugar, methyl 2:4-di-O-methyl 3:6-anhydro- -L-galactoside, was isolated (23). The anhydro-sugar was thought to have arisen by alkaline scission, during methylation, of a sulphate residue situated at C_6. However it was pointed out, by Percival et al., that the amount of anhydro-sugar isolated was far in excess of that which would be required by a sulphate content of ca. 1%; and so it was held by these investigators that 3:6-anhydro-L-galactose was an integral part of the agar molecule (24).

Later experiments pointed to the existence of at least two polysaccharides in agar extract (25). One of these, agarose, on methanolysis afforded the dimethyl-acetal of 3:6-anhydro-L-galactose in 20% yield, thus verifying the contention that this residue is present in the agar molecule. Partial methanolysis afforded 45% yield of a disaccharide, agarobiose, comprising D-galactose glycosidically linked to C_4 of 3:6-anhydro-L-galactose; the β-configuration was assumed for the disaccharide in view of its low specific rotation.



Structure of Agarose

On the other hand, enzymic hydrolysis of agarose afforded 28% of a disaccharide with a high positive rotation, neo-agarobiose, in which 3:6-anhydro-L-galactose is glycosidically linked to C_3 of D-galactose (26).

Agarose then consists of alternate 1:3-linked β-D-galactopyranose units and α-1:4-linked 3:6-anhydro-L-galactopyranose units. It is believed that the agarose chain is terminated at the non-reducing end by a 3:6-anhydro-L-galactose residue, and at the reducing end by a D-galactose unit; neither D-galactose nor the anhydro-sugar was detected in the enzymic hydrolysate, which involved cleavage of β-glycosidic links.

The other polysaccharide separated from agar, agaropectin, has not been investigated in detail. It is assumed to be of more complicated constitution, containing uronic acid and sulphate, as well as D-galactose and 3:6-anhydro-L-galactose residues (27).

The above experiments were carried out by Japanese workers on agar of unknown origin and purity. Other workers have investigated the agar extract from Gelidium cartilaginum (28); from analytical results and a study of the mercaptolysis products, it was concluded that this material consisted of alternating D-galactose and 3:6-anhydro-L-galactose units, with a half-ester sulphate grouping on every tenth galactose unit.

(b) Galactan from Dilsea edulis

Dilute acid extraction of the red weed,

Dilsea edulis, affords a polysaccharide containing 70% of D-galactose, together with 10% of D-glucuronic acid, 7% of D-xylose, and 12% of organically-bound sulphate (30). Similar results were obtained from earlier work on the related red weed, Dumontia incrassata (29). Methylation results on the Dilsea extract, together with investigation by the Barry degradation procedure, have indicated that the main chain of the molecule consists of 1:3-linked galactopyranose units; it was further concluded that glucuronic acid residues, together with 1:3-linked xylopyranose units, occur around the periphery of the molecule.

(c) Carrageenin

Carrageenin is the aqueous extract of the red weed, Chondrus crispus, and of the closely-related weeds, Gigartina stellata and Chondrus oceallatus, all of the Gigartinaceae family. It contains 30% of organically bound sulphate; D-galactose accounts for two-thirds of the organic material, while L-galactose and 3:6-anhydro-D-galactose comprise the remainder (31). Reactions characteristic of keto-sugars, which have been reported for carrageenin, are now ascribed to the anhydro-sugar (32),

It has recently been found that carrageeni consists of at least five polysaccharides (33). Fractionation, based on the gelatinising effect of the potassium ion, has led to the separation of two main components: the κ-fraction contains a large proportion of 3:6-anhydro-D-galactose units, while on the other hand λ-carrageenin is composed mainly of

D-galactose-4-sulphate residues, together with a trace of 3:6-anhydro-D-galactose.

Partial mercaptolysis of κ-carrageenin has led to the isolation of a disaccharide derivative containing D-galactose glycosidically linked to C_4 of 3:6-anhydro-D-galactose (34). The proposed structure for the κ-carrageenin molecule envisages a chain of 1:3-linked disaccharide units, with a point of branching at every fifth galactose unit. Since the molecule consumes no periodate, it has been suggested that D-galactose disulphate residues may be present as end-groups (35). These investigations on κ-carrageenin point to its essential similarity with agarose, except that the anhydro-sugars are of different configuration :

```
     GSS
     :
     6
....3GS1....4A1....3GS1....4A1....3GS1....4A1....3GS1....4A1.....
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GS = D-galactopyranosyl sulphate; A = 3:6-anhydro-D-galactopyranose

GSS = D-galactopyranose 3:4 or 3:6 disulphate

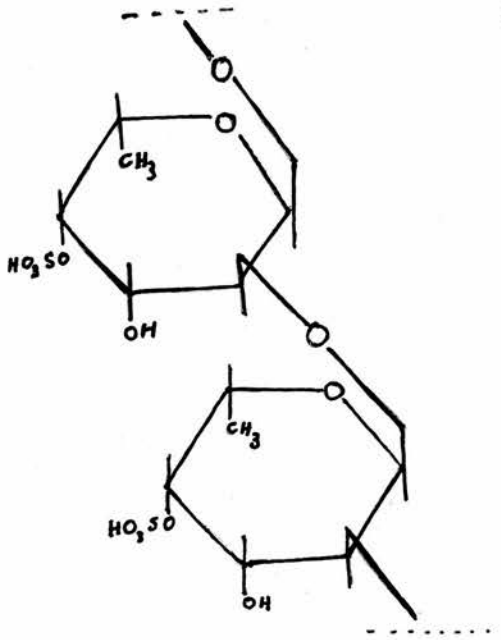
Structure of κ-Carrageenin

Methylation studies on a degraded resistant residue of carrageenin have led to the formulation of a structure containing a 1:3-linked backbone of D- and L-galactose units, with alkali-resistant sulphate groupings situated at C_4 (36). The presence of branch-points at C_6 is also indicated, but, as in the case of agar, more investigation is needed before a detailed structure of the molecule can be advanced.

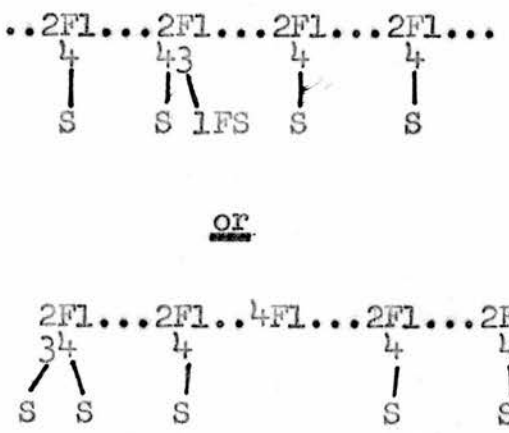
Methylpentosans

L-rhamnose has been reported among the hydrolysis products of several polysaccharides of the green seaweeds, but always in conjunction with other residues such as D-xylose and D-glucose. L-fucose, in the form of its sulphated polymer fucoidin, is a common constituent of the brown weeds; various small amounts of other sugars which have been reported as occurring in the polysaccharide are now ascribed to admixture of the original material with other weeds or polysaccharides (37).

The amount of sulphate in the polysaccharide (33%) is that required for one ester grouping per anhydrosugar unit. The stability of the sulphate group to alkaline hydrolysis, as in the case of carrageenin, makes it very probable that the ester groupings are situated at C_4. Sulphate esters with a trans-hydroxyl group on the adjacent C-atom are readily hydrolysed by alkali (38), and therefore the possibility of location at C_3 or C_2 can be eliminated in the case of fucoidin. Methylation afforded 3-O-methyl-L-fucose, 2:3-di-O-methylfucose, and free L-fucose; the relative proportions of these sugars indicated that the typical repeating unit of fucoidin is a 1:2-linked fucose residue, with the sulphate on C_4 (39). Recent isolation of a disaccharide derivative , linked in the 1:2-position, fron the products of partially hydrolysed reduced fucoidin, have confirmed these conclusions (40),(cf. over).



repeating unit



general structure

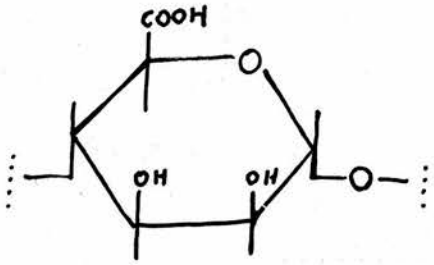
Fucoidin

Polyuronides

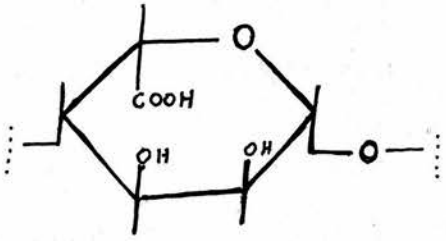
Although D-glucuronic acid has been reported in the red seaweed Dilsea edulis (cf. above), and in the green weed Ulva lactuca (cf. below), the characteristic polyuronide of the brown seaweeds is a polymannuronic acid, algin. It is extracted in high yield from the brown seaweeds by dilute sodium carbonate solution, the amount present in the plant being related to the laminarin and mannitol content (4).

Complete hydrolysis of alginic acid requires drastic conditions, and the yield of D-mannurono-lactone has never exceeded 35% (41). Methylation of the polysaccharide led to the isolation of 2:3-di-O-methyl-D-mannuronic acid, thus establishing that at least some of the linkages in the molecule were of the 1:4 variety (42). This conclusion was later verified by the isolation in high yield of 2:3-di-O-methylmannose from the reduction of methylated, hydrolysed, degraded alginic acid (43).

The reported isolation of L-guluronic acid has led to the possibility that two sugar residues are present in the molecule (44). The presence of guluronic acid has received further confirmation by the isolation of L + tartaric acid from the hydrolysate of periodate and bromine-oxidised alginic acid (45).

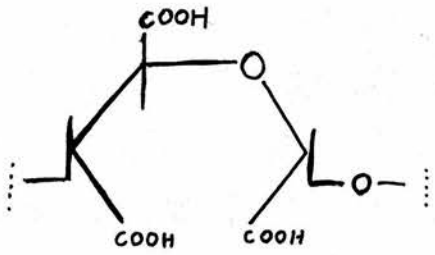


1:4-D-Mannuronic acid

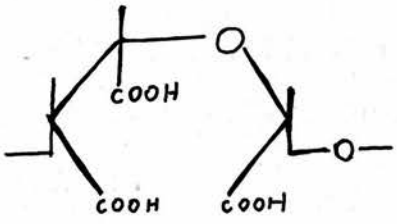


1:4-L-Guluronic acid

IO_4^- + Br_2

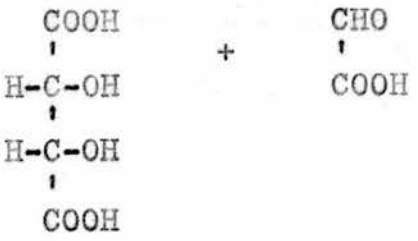


IO_4^- + Br_2

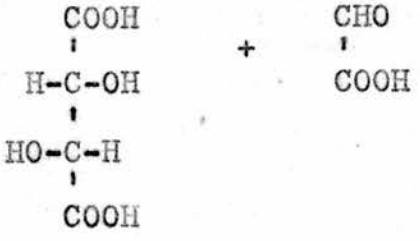


Hydrolysis

Hydrolysis



Meso-tartaric acid



L + Tartaric acid

Polysaccharides Containing Several Sugar Residues

With the exception of the extract from Dilsea edulis, the polysaccharides described above consist of either one sugar residue, or of several stereoisomeric derivatives of the same sugar. The extracts of the green weeds described below are characterised by the presence of several sugar residues, together with a relatively high sulphate content (ca. 20%).

The Polysaccharide Extract from Cladophora rupestris (46)

Hot-water extraction of the green seaweed, Cladophora rupestris, afforded a water-soluble polysaccharide in 11.5% yield. The isolated material was contaminated by the presence of protein (ca. 20%), which was largely removed by treatment with trichloracetic acid. The purified material (6.5% yield) had $[\alpha]_D$ + 69°, and a sulphate content of ca. 20%. Estimation of the sulphate present in the ash, which was half the amount present in the polysaccharide, confirmed that the sulphate was ethereally linked (21). These residues, like the sulphate groups in the galactans previously discussed, proved to be stable to alkali. Uronic acid estimation, by the decarboxylation method, gave a value of less than 5%.

Hydrolysis of the polysaccharide and estimation of the relative proportions of the constituent sugars gave :-

L-arabinose	D-galactose	D-xylose	L-rhamnose	D-glucose
3.7	2.8	1.0	0.4	0.2

Each of these sugars was separated

and characterised by the formation of authentic crystalline derivatives. Partial hydrolysis studies gave evidence for the presence of contiguous arabinose units, oligosaccharides containing only galactose or xylose being also separated. No evidence was obtained that glucose was linked to any other sugar residues in the macro-molecule.

This finding was supported by the isolation of a chloroform-soluble, acetylated, glucose-rich fraction. The low periodate consumption of this material after deacetylation (1 mole per 420 g.), together with its hydrolysis by laminarinase, indicated its essential similarity with laminarin. Methylation of the residual chloroform-insoluble acetate afforded a product with methoxyl content of 25.1%. Hydrolysis and chromatographic separation of the methylated sugars indicated that some of the main linkages in the polysaccharide were :-

Galp1.... ...6Galp1... ...6Galf1... $\begin{matrix}\cdot\cdot\cdot^{3}\\ \cdot\cdot\cdot_{6}\end{matrix}$Gal1...

$\begin{matrix}\cdot\cdot\cdot^{4}\\ \cdot\cdot\cdot_{6}\end{matrix}Gal\begin{matrix}1\cdot\cdot\cdot\\ 3\cdot\cdot\cdot\end{matrix}$ $\begin{matrix}\cdot\cdot\cdot^{4}\\ \cdot\cdot\cdot_{6}\end{matrix}Gal\begin{matrix}1\cdot\cdot\cdot\\ 2\cdot\cdot\cdot\\ 3\cdot\cdot\cdot\end{matrix}$ ...3Ap1... ...$\begin{matrix}3\\ 4\end{matrix}$Ap1...

$\begin{matrix}\cdot\cdot\cdot^{2}\\ \cdot\cdot\cdot_{3}\end{matrix}$Ap1... X1... ...4X1... ...$\begin{matrix}3\\ 2\end{matrix}$R1...

Galp = galactopyranose; Galf = galactofuranose; Ap = arabopyranose; X = xylopyranose; R = rhamnose

Although most of the standard means of fractionation were attempted, all efforts to separate the extract into several polysaccharides were unsuccessful. However, it appears probable that more than a single entity is present.

The Polysaccharide Extract from Ulva lactuca (47)

Sodium carbonate extraction of the green seaweed, Ulva lactuca, afforded polysaccharide material in 18% yield. The extract was contaminated with protein (ca. 25%); although treatment by the Sevag process removed the nitrogenous material, a considerable amount of polysaccharide was lost at the same time. The purified polysaccharide had $[\alpha]_D$ - 47°; sulphate 17.5%; uronic anhydride 20.8%.

Methanolysis and hydrolysis of the polysaccharide, followed by estimation of the relative sugar proportions, gave :-

L-rhamnose	:	D-xylose	:	D-glucose
4.4	:	1.3	:	1.0 .

No positive evidence as to the nature of the uronic acid residues was obtained, although indications of the presence of D-glucuronic acid were encountered. The periodate consumption of the material was 1 mole per 960 g., indicating that one unit in five had contiguous hydroxyl groupings.

Methylation of the polysaccharide and extraction of a chloroform-soluble portion yielded a material with 31% methoxyl content, and with relatively low sulphate (ca. 2.5%). Methanolysis of this polysaccharide indicated the presence of 2:3:4-tri-O-methylrhamnose, 2:3:6-tri-O-methyl-glucose, and 2;3-di-O-methylglucose.

Investigation of the hydrolysis products of the chloroform-insoluble fraction (OMe 17%) gave evidence of the presence of 2:3:4-tri- and 2:3-di-O-methylxylose,

2:3:4-tri- and 2:3-di-O-methylrhamnose, free rhamnose and free xylose. An unidentified mono-O-methylrhamnose fraction was also isolated.

Summary

On the basis of these investigations, it appears that water extraction of the green seaweeds affords a complex extract which is characterised by the presence of several sugar residues. Both extracts from Cladophora and from Ulva are accompanied by a relatively high proportion of protein contaminant, and the pure polysaccharide contains ca. 20% of combined sulphate. Arabinose, galactose and xylose are the main sugars in the Cladophora extract, together with minor amounts of glucose and rhamnose; the material from Ulva has rhamnose, xylose, glucose and uronic acid.

Differential solubilities of the polysaccharide acetate and methylate in each case led to the separation of a non-sulphated, glucose-rich fraction, indicating that in neither instance is the sulphate combined with glucose units. Methylation by the Haworth method in each case led to a product with relatively low methoxyl value, and containing a high proportion of unmethylated sugars.

Because of the variation in the carbohydrate constitution of these green weeds, a structural examination of the polysaccharide components of Acrosiphonia centralis (Spongomorpha arcta) was carried out as described below. This green seaweed is a close botanical relative of Cladophora

rupestris, the extract from which has been exhaustively described above. This investigation is part of a larger programme, wherein the polysaccharide constituents of each of the main families of the Chlorophyceae will be examined. It is possible that such work might provide correlations between polysaccharide identity and botanical similarity.

Experimental

EXPERIMENTAL

General Methods Used

Solutions were concentrated at 45°/15 mm.

Rotations were determined in water, unless otherwise stated.

Isolation : The polysaccharide was obtained either by freeze-drying, or by precipitation from concentrated solution by addition of ethanol. In the latter case, the precipitated material was dried by repeated washing with ethanol and ether.

Ash determinations were carried out by ignition of 50-mg. samples in a platinum crucible until constant weight was attained.

Sulphate determinations were carried out by heating 50-mg. samples at 100° in 8 N hydrochloric acid (containing a few drops of nitric acid) over five hours. Addition of 3% barium chloride to the filtered solution was followed by gravimetric estimation of the precipitated barium salt. (51).

Nitrogen analyses were carried out by a semi-micro modification of the Kjeldahl method.

Methoxyl determinations were carried out by a micro-modification of the Zeisel method.

Demethylations were carried out by heating 5 mg. samples with hydrobromic acid for ten minutes, followed by neutralisation with silver carbonate and evaporation to dryness. Chloroform extraction of the residual salts gave the demethylated sugar. Hydriodic acid was used to demethylate rhamnose derivatives.

Dialysis of the polysaccharide in solution was carried out

against a closed system of distilled water, which was frequently changed over three days. The efficacy of this procedure was proved by dialysis of a test solution of the polysaccharide in 10% barium chloride, no trace of the inorganic material being left inside the dialysis tubing after the second day.

Cellulose columns were prepared by dry-packing; small columns were prepared by pouring in a slurry of cellulose in acetone in small portions at a time.

Light petroleum was of b.p. 60-80°, unless otherwise stated.

Thick paper separations were carried out on Whatman No. 3 paper; in all cases the paper was subjected to preliminary extraction with a 1:3 mixture of benzene:ethanol.

Chromatography was carried out on Whatman No. 1 paper, using one of the following systems :-

1 - Benzene : n-butanol : pyridine : water :: 1 : 5 : 3 : 3

2 - Ethyl acetate : pyridine : water :: 10 : 4 : 3

3 - Ethyl acetate : acetic acid : formic acid : water ::18:4:1:5

4 - Ethyl acetate : acetic acid : water :: 3 : 1 : 3

5 - n-Butanol : acetic acid : water :: 4 : 1 : 5

6 - n-Butanol : ethanol : water :: 4 : 1 : 5

7 - Methyl ethyl ketone, half-saturated with water containing 1% ammonium hydroxide.

8 - Ethyl acetate : acetic acid : water :: 9 : 2 : 2 .

Where these solvent systems form two layers, in all cases the upper layer was used. The following abbreviations are used to describe relative mobilities of the sugars on paper chromatograms :-

R_G refers to the rate of movement of the sugar relative to that

of tetra-O-methylglucose.

R_{Gl} refers to the rate of movement relative to that of glucose.

$R_{G.A.}$ refers to the rate of movement relative to that of glucuronic acid.

R_F refers to rate of movement relative to that of the solvent front.

Ionophoresis was carried out at a potential of 500 volts, in borate buffer at pH 10, or in acetate buffer at pH 5.5. The chromatograms were sprayed with a saturated solution of aniline oxalate in water containing 5% acetic acid. (48)

Investigation of Different Methods of Extraction

All preliminary cleansing of the Acrosiphonia weed was carried out with sea-water, thus reducing to a minimum any loss of water-soluble polysaccharide. All samples were air-dried and macerated before extraction. The dry weed was always subject to a preliminary extraction with 85% aqueous ethanol to remove readily-soluble lipids, colouring-matter, free sugars, etc.

(a) - Hot Water Extract - Acrosiphonia centralis weed (collected at Millport, April 1957) was stirred at 100° with water (1 litre) for one hour. The viscous solution was filtered through muslin and celite, the weed (100 g., dry weight) being extracted five times in all. Evaporation of the extracts to small volume, followed by dialysis and freeze-drying, afforded polysaccharide material as a grey-white powder (4.63 g., 4.6%).

(b) - Hydrochloric Acid Extract - The residues from the hot-water extract were stirred overnight in 0.01 N hydrochloric acid (400 ml.) and the polysaccharide solution separated by filtration. The procedure was repeated; the combined extracts after dialysis and freeze-drying afforded 0.42 g. of polysaccharide (0.4%).

(c) - Sodium Carbonate Extract - The residual weed from previous extractions was stirred overnight with 2% sodium carbonate solution, and the polysaccharide isolated as before (0.29g.,0.3%).

(d) - Hydrolysis of the Weed Residues - A sample of the residual

weed (10 g.) was allowed to stand for ten days in 72% sulphuric acid solution (49). The solution was then filtered and diluted to a strength of 1 N. Hydrolysis for seven hours at 100°, followed by chromatographic examination of the neutralised hydrolysate, revealed the presence of approximately equal proportions of glucose, xylose and rhamnose (the constituent sugars of the water-soluble material). It was therefore decided to investigate further methods of extraction.

(e)-- Potassium Hydroxide Extract - Aqueous-extracted weed (25 g.) was stirred with 5% potassium hydroxide solution over four hours, the extraction being repeated. After neutralisation and dialysis, the polysaccharide was isolated by alcohol precipitation (0.12 g., 0.5%).

(f) - Ammonium Oxalate Extract - Acrosiphonia centralis (25 g.) was exhaustively extracted with 1% ammonium oxalate at 100°; the combined filtrates from nine successive extractions were dialysed and evaporated to small volume. Addition of four volumes of ethanol gave polysaccharide material as an off-white powder (3 g., 12%).

(g) - Further Hydrolysis of the Weed Residues - Treatment of the material remaining after ammonium oxalate extraction, as described above (d), revealed that glucose, xylose and rhamnose were still present in equal amount; it thus appears that these sugars form an integral part of the skeletal structure of the Acrosiphonia centralis weed.

(h) - Attempted Detection of Cellulose - Both the polysaccharide and the residual weed were treated with Schwiezer's cuprammonium reagent, a control experiment being carried out on commercial cellulose (50). Although the cellulose dissolved to give a gelatinous solution, and could be reprecipitated on acidification, neither the polysaccharide nor the weed residues were appreciably affected by the reagent.

Treatment of the polysaccharide and residues with Nowoprofsky's reagent afforded no appreciable colour change. Addition of cellulose to the reagent resulted in a change of colour from orange to deep blue; it was thus again indicated that cellulose is not present in the Acrosiphonia weed in large amount.

Trial Hydrolysis of the Polysaccharide

A sample of the hot-water extract (0.335 g.) was heated with N sulphuric acid (30 ml.) at 100°. The course of hydrolysis was followed, on aliquots withdrawn at intervals, by chromatographic examination, and by measurement of specific rotation and reducing power (iodine uptake).

Time (hr.)	0	0.5	1	2	4	6	8
I_2(0.01 N,ml.)	1.9	5.4	7.1	8.6	9.7	-	-
$[\alpha]_D$	-29°	-12°	-4°	+2°	+6°	+4°	+6°

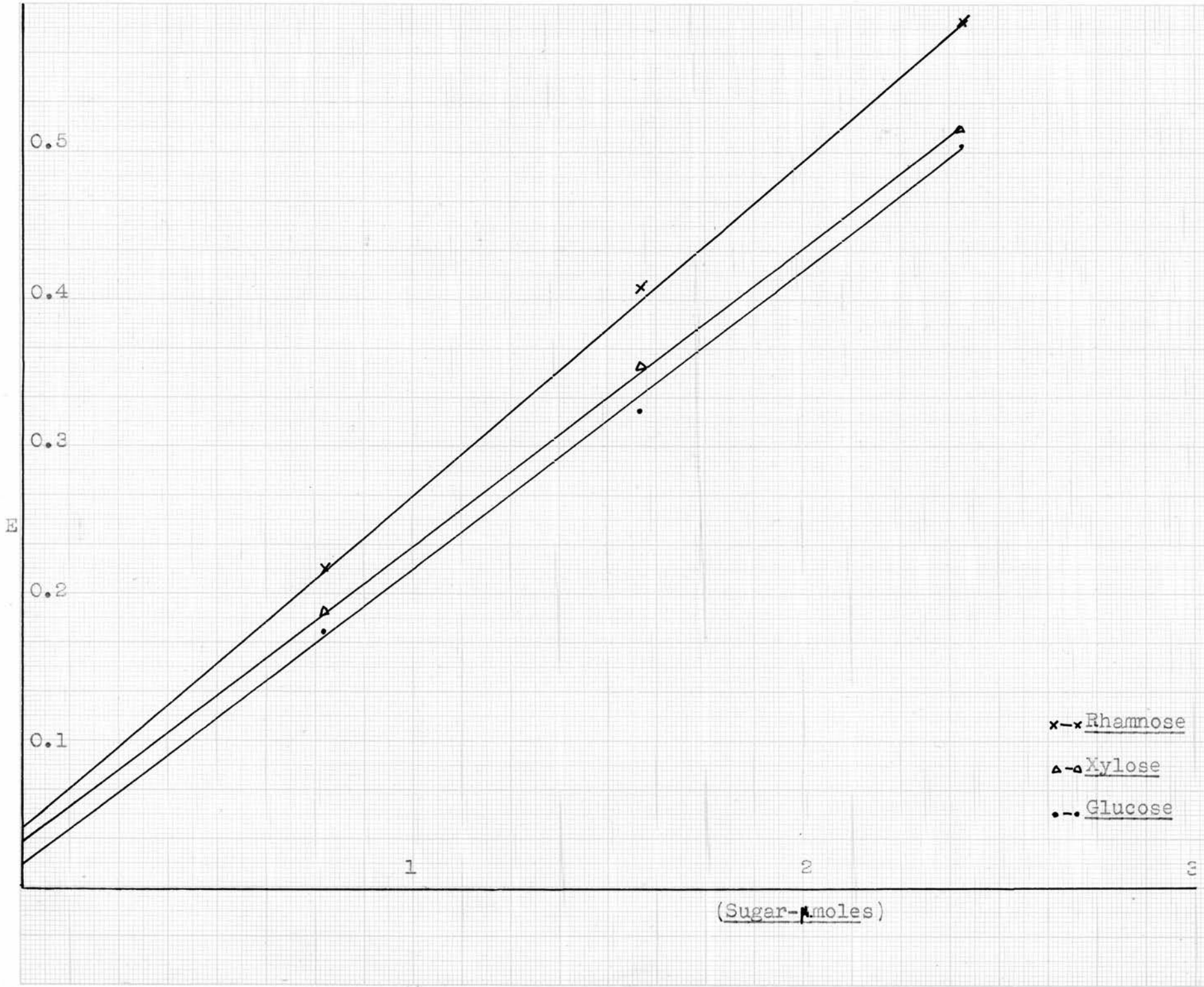
It was thus seen that after about 5 - 7 hours, degradation as well as hydrolysis was taking place. Chromatographic examination revealed that glucose, xylose and rhamnose were released within

the first hour, accompanied by traces of neutral oligosaccharides; traces of oligosaccharides were still visible after six hours, but had entirely disappeared at eight hours. The presence of uronic acids was indicated by a series of pink spots (purple under U.V. light) near the starting-line. Trace quantities of mannose and galactose were also visible.

On the basis of these results, it was decided that all polysaccharide hydrolyses, unless otherwise stated, would be carried out as follows: The polysaccharide was heated in N sulphuric acid at 100° for seven hours (c. 10 g. per litre). The cooled hydrolysate was neutralised with barium carbonate, filtered, and the precipitated salts washed exhaustively with water. Evaporation of the combined filtrate and washings to dryness was followed by water extraction of the resultant solid. Filtration of the solution and evaporation afforded polysaccharide as a white solid.

Determination of the Relative Proportions of the Sugars Present in the Different Extracts (52)

A synthetic mixture of the sugars present in the hydrolysate was made up by dissolving millimolar quantities of these sugars in 25 ml. of water. Measured quantities of this mixture (0.02, 0.04, 0.06 ml.) were applied from an Agla micro-burette at intervals along the starting-line of a paper chromatogram (20 x 40 cm.). Also spotted on were samples of the hydrolysate of the hot-water extract (a).


0.5
0.4
0.3
E
0.2
0.1
1
2
3
(Sugar-µmoles)
×—× Rhamnose
△—△ Xylose
•—• Glucose

The chromatogram was eluted for four days (solvent 1) and allowed to dry in an atmosphere of acetic acid at room temperature. It was then sprayed with a 1% solution of freshly-prepared p-anisidine hydrochloride, and heated at 110° for ten minutes. Each of the coloured spots which developed was cut out and left to stand with 3 ml. of methanol for ten min. The density of the resultant solution was then measured in a Unicam Spectrophotometer at the wavelength of maximum absorbance for the particular sugar being examined. When the readings for the standard solutions of the respective sugars are plotted against concentration, a straight-line graph is obtained in each case. It is then possible to read off from the graph the concentrations corresponding to the absorbances of the sugars in the hydrolysate.

Sugar	Rhamnose	Xylose	Glucose
λ(mμ)	385	510	410
Standard soln(0.02ml.)	0.225	0.18	0.19
Hydrolysate (a)	0.18	0.27	0.12
Standard soln(0.04ml.)	0.41	0.33	0.36
Hydrolysate (b)	0.32	0.49	0.21
Standard soln(0.06ml.)	0.59	0.51	0.52

The straight-line relationship between concentration and absorption of the sugars in the standard solutions is detailed opposite, together with the equivalent concentrations of the hydrolysate sugars.

A complete duplication of the experiment ensured accuracy to within ± 10%. The final results for the hot-water extract are as follows : -

Glucose	Xylose	Rhamnose
1.0	2.5	1.3

The relative molar proportions of the sugars present in the various other hydrolysates were similarly determined :-

Extract	Glucose	Xylose	Rhamnose
HCl (b)	1.00	0.26	0.28
Na_2CO_3 (c)	1.00	2.0	1.5
Amm.Ox. (f)	1.00	2.1	1.3

In view of the similarity between the hot-water and ammonium oxalate extracts, and because of the higher yield by the latter procedure, it was decided that all future investigations would be carried out on this material. Before doing so, a more detailed comparison between the two materials was carried out.

Estimation of Sugars by Periodate Oxidation (53)

The polysaccharide (53.1 mg., ammonium oxalate extract) was hydrolysed with N acid at 100° for seven hours, affording 39.8 mg. of neutralised hydrolysate. The solid was dissolved in water (2 ml.) and 0.3 ml. of the solution applied along the starting-line of a paper chromatogram (20 x 40 cm.). Side strips were spotted with a sample of the hydrolysate to locate the bulk of the sugars after elution. After running for two days (solvent 3), the chromatograms were dried in vacuo.

Sections of paper containing the different sugars (detected by spraying marginal strips) were then cut off and exhaustively extracted with water (54). Treatment with sodium periodate (0.2 M, 3 ml.) at 100° for 0.5 hr. was followed by addition of ethylene glycol. The formic acid in solution was then estimated by titration against 0.01 N sodium hydroxide solution.

Sugar	NaOH(ml.,0.01N)	Sugar (mg.)	% of syrup	Molar props
Galactose	0.19	0.07	1.2	0.12
Glucose	1.68	0.61	10.2	1.00
Mannose	0.31	0.12	2.1	0.21
Xylose	2.82	1.05	17.6	2.2
Rhamnose	2.32	0.96	16.1	1.6
		2.81 mg.	47.2%	

A similar estimation of the hydrolysate from the hot-water extract gave the following results :-

Sugar	Galactose	Glucose	Mannose	Xylose	Rhamnose
% of syrup	1.1	10.9	1.3	22.5	15.1
Molar proportions	0.10	1.00	0.12	2.5	1.5

It was later shown that about 50% of the polysaccharide hydrolysate consisted of the barium salts of a mixture of oligo-uronic acids.

General Properties of the Two Extracts

Percentage protein was estimated by multiplying the nitrogen content by 6.25. In the case of the ammonium oxalate extract, nitrogen present as the ammonium salt of the polysaccharide was determined by a direct distillation

with alkali; the amount obtained (3.6%) was then subtracted from the total Kjeldahl estimation.

Extract	%Ash	%Sulphate	%Protein	$[\alpha]_D$	(c.)
Hot-water	13.9	7.8	6.4	-45°	0.5
Amm.Ox.	10.0	7.8	3.5	-31°	1.2

Further analyses on the polysaccharide extracted by ammonium oxalate gave :- Sulphated ash, 11.8%; Sulphate in ash, 32%, i.e. 3.8% of the polysaccharide. All subsequent work was carried out on this material.

Equivalent Weight Determination

The polysaccharide (133 mg.) was dissolved in water (20 ml.), and the solution recycled four times through a column of Amberlite IR 120 H resin (10 g.). The resultant acid solution (pH 2-3) was freeze-dried to a white powder (Found: Ash, 0.0; SO_4, 7.5%). The equivalent weight was determined by dissolving samples (20 mg.) in water (5 ml.), and titrating against 0.01 N sodium hydroxide solution (phenolphthalein indicator). The mean of several determinations gave a value of 459 g. for the free-acid polysaccharide.

The uronic anhydride content of the neutral polysaccharide, as estimated by the decarboxylation method (55), was 20.3%.

Attempted Fractionation of the Polysaccharide

Experiments with Cetavlon

The polysaccharide (200 mg.) was dissolved in water (20 ml.) and a 10% aqueous solution (5 ml.)

of cetyl trimethyl ammonium bromide ("Cetavlon") was added dropwise with stirring. The resulting precipitate was removed by centrifugation, washed with water, and then redissolved in 2 N acetic acid (10 ml.). Reprecipitation by addition of ethanol, followed by washing with absolute ethanol and ether, afforded the Cetavlon-precipitated polysaccharide as a white powder (130 mg.). The supernatant liquid from the initial precipitation was poured into four volumes of ethanol, and the precipitated polysaccharide (40 mg.) isolated and dried as before.

Four successive fractionations in all were carried out; in each case hydrolysates of both complexed and supernatant material revealed the presence of glucose, xylose and rhamnose, with no significant difference in their relative proportions.

1/ - 189 mg. of polysaccharide gave 132 mg. of precipitated material, and 37 mg. from the soluble fraction. Found; SO_4'', soluble material, 9.5%; insoluble material, 8.1%.

2/ - 347 mg. of polysaccharide were treated as above; both fractions were purified by dissolution and reprecipitation four times, but once again no obvious difference in the sugar content of the fractions was observed. (Yield of insoluble material, 262 mg.: yield of insoluble material 23 mg.).

3/ - 138 mg. of polysaccharide dissolved in borate buffer at pH 10 gave 107 mg. of precipitated material; addition of ethanol to the supernatant liquid afforded no precipitate.

4/ - 97 mg. of polysaccharide, dissolved in 0.1 M NaOH, (10 ml.), yielded 69 mg. of insoluble material; addition of ethanol to the supernatant afforded no further precipitation.

Experiments with Copper Salts

As in the case of Cetavlon, chromatographic examination of the hydrolysed fractions revealed that no appreciable fractionation had occurred.

1/ - A solution of the polysaccharide (107 mg.) in water (10 ml.) was treated with a 10% solution (2 ml.) of copper sulphate. The precipitated material was centrifuged, washed with water, and with a 10% solution of hydrochloric acid in methanol. Further treatment with ethanol and ether afforded a white powder (18 mg.).

2/ - 107 mg. of polysaccharide, treated similarly with 5% copper acetate solution (5 ml.), yielded 23 mg. of insoluble material. Addition of ethanol to the supernatant liquids of both fractionations gave no polysaccharide material, no change occurring on acidification of the solution.

3/ - 88 mg. of polysaccharide afforded 27 mg. of precipitate on addition of copper acetate solution. The supernatant liquid was made acid, dialysed, and the residual polysaccharide isolated by freeze-drying (34 mg.).

Oxidation of the Polysaccharide

The polysaccharide (0.153 g.) was dissolved in water (30 ml.) and an equal volume of sodium periodate solution (0.095 M) was added. The uptake of oxidant and production of acid were measured on 1 ml. samples as described for the oxidation of laminarin (Part II, experimental).

2.5 ml. of arsenite soln. (0.05 N) = 21.9 ml. of iodine soln. (0.0114 N).

2.5 ml. of arsenite soln. + 0.5 ml. of periodate soln. (0.095 M) = 5.1 " " " " .

Time (hr.)	1.25	3.5	8	25	32	48	96
Iodine soln. (0.0114 N - ml.).	6.8	8.9	10.1	12.9	14.2	15.7	17.4
Alkali (0.01 N - ml.).	0.82	1.05	1.27	1.48	1.52	2.35	3.46

After 96 hours :-

Periodate consumed = 1 mole per 122 g. of polysaccharide

Acid produced = 1 " " 249 g. " " .

Oxidation was then stopped by passing sulphur dioxide through the cooled reaction mixture. After dialysis, freeze-drying afforded oxo-polysaccharide as an off-white powder (204 mg., 68%). In a repeat experiment carried out under the same conditions, a value of one mole of periodate per 120 g. of polysaccharide was arrived at; the amount of acid produced was found to be one mole per 209 g..

Examination of the Oxopolysaccharide

A sample (42.6 mg.) of the oxidised material (found: Ash, 6.7; SO_4'', 8.6%) was hydrolysed for seven hours (N H_2SO_4), affording 30.2 mg. of neutralised hydrolysate. Chromatography indicated that xylose and rhamnose were present in very small amount. A sample of the oxidation solution, which was not dialysed but treated with ion-exchange resins to remove inorganic material, gave a similar hydrolysate.

Estimation of the sugars present gave (cf. page 29) gave :-

Rhamnose 2.4%

Xylose 1.9%

When the time of hydrolysis was increased to twenty-one hours, a slight increase in the amount of rhamnose was observed.

Characterisation of the Individual Sugars

The polysaccharide (2.95 g.) was hydrolysed (cf. page 26), affording 1.75 g. of neutralised hydrolysate; this material was applied to a cellulose column (2.8 x 60 cm.), and eluted with n-butanol half-saturated with water. Fractions (7-ml.) were collected at ten-minute intervals, every tenth tube being evaporated and analysed chromatographically. Like fractions were combined and evaporated to dryness; before weighing, purification was effected by dissolving in water, filtering and evaporating to dryness in a weighed flask. The total yield from the column was thus found to be 79%. R_F-values are quoted for solvent 1/.

Fraction 1 : 28.8 mg.; R_F 0.69

$[\alpha]_D$ - 4°, (c. 2.9)

The syrup was chromatographically identical with 3-O-methylrhamnose (solvent 5/), and gave a yellow colour with aniline oxalate spray, (found: OMe 0%). The substance gave a positive Seliwanoff test.

Fraction 2 : 139 mg.; R_F 0.55

Further purification yielded a crystalline material of m.p. and mixed m.p. with L-rhamnose hydrate 68°. The derived crystalline benzoylhydrazone had m.p. and mixed m.p. 186°.

Fraction 3 : 18.5 mg.; R_F 0.46

The syrup, which gave a pink colour with aniline oxalate spray, was chromatographically distinct from ribose, and gave a positive Seliwanoff test, indicating degradation-product.

Fraction 4 : 264.0 mg.; R_F 0.44

$[\alpha]_D$ + 19°, (c. 1.0)

Nucleation of the clear syrup afforded crystalline D-xylose, of m.p. and mixed m.p. 142°. The derived dibenzylidene dimethyl acetal had m.p. 186°.

Fraction 5 : 39.0 mg.; R_F 0.38

Although slightly contaminated with xylose, the syrup was chromatographically identical with mannose (solvents 1 and 3). Treatment with phenylhydrazine in acetic acid solution at 0° afforded a crystalline phenylhydrazone of m.p. 188°. The melting-point was undepressed by admixture with an authentic specimen of similar m.p., prepared by the same method.

Fraction 6 : 56.6 mg.; R_F 0.33

$[\alpha]_D$ + 53°, (c. 0.25)

The identity of the syrup as D-glucose was proved by formation of the crystalline dichloro-

phenylhydrazone derivative, with m.p. and mixed m.p. 153°.

Fraction 7 : 20.9 mg.; R_F 0.28

The syrup was chromatographically identical with galactose (solvents 1 and 3). Identity was conclusively proved by formation of the crystalline diethyl-mercaptal; the material had m.p. 140-2°, undepressed by admixture with the diethyl-mercaptal of D-galactose.

Fraction 8 : 826 mg. ; R_F 0 - 0.07

The material, eluted by water from the cellulose column, consisted of the barium salts of a mixture of uronic acids. It was dissolved in a minimum of water, and treated with IR-120-H resin. Evaporation of the filtrate and washings gave a yellow acidic syrup (0.61 g., pH 2); this material was stored at 0°C, since on standing at room temperature it tended to decompose. Chromatography in acidic solvents indicated a mixture of oligouronic acids.

Preliminary Separation of an Aldobiuronic Acid

Fractionation of the mixture on a cellulose column (solvent, ethyl acetate : acetic acid : water :: 9 : 2 : 2) afforded a chromatographically pure syrup (129 mg.). The material had $[\alpha]_D$ - 8° (c. 1.2); $R_{Gl.}$ 1.05 (solvent 3); $R_{G.A.}$ 0.85 (solvent 4). Titration of a sample against 0.02 N sodium hydroxide solution , using phenolphthalein indicator, gave an equivalent weight of 367 g. (calculated for glucuronosyl-O-rhamnose, 340 g.).

The syrup (45.6 mg.) was refluxed for six hours with 2% methanolic hydrogen chloride (5 ml.), and then neutralised with silver carbonate. Exhaustive extraction of the residues with methanol, followed by evaporation to dryness, yielded the ester glycoside as a pure syrup (47.4 mg.).

The material was suspended in tetrahydrofuran (5 ml.), which had been carefully dried by distillation from sodium and from lithium aluminium hydride. A saturated suspension of the hydride in tetrahydrofuran was then added dropwise (1 ml.), and the solution refluxed for one hour. Addition of water (3 ml.) was followed by filtration of precipitated aluminium hydroxide, and treatment with IR-120-H resin to remove cations in solution. After evaporation of the organic liquid, an equal volume of 2 N sulphuric acid was added to the remaining aqueous solution, and the mixture (10 ml.) heated at 100° for six hours. Deionisation with IR-4-B resin, followed by evaporation to dryness and methanol extraction of the residues, afforded reduced hydrolysed biuronic acid as a clear syrup (33.7 mg.). Chromatography (solvents 1 and 3), together with colorimetric estimation, revealed the presence of glucose and rhamnose in the molar proportions of 1 : 0.95.

The hydrolysed material (30 mg.) was treated with glucose oxidase, an enzyme which catalyses the oxidation of glucose to the non-reducing gluconic acid (56). After standing for two days, the enzyme was removed by addition of equivalent solutions of cadmium sulphate and barium hydroxide, and filtration of the resultant precipitate. Evaporation

followed by methanol extraction yielded a clear syrup which contained only rhamnose (paper chromatography). The identity of the sugar was confirmed by formation of the benzoylhydrazone derivative, with m.p. and mixed m.p. 185°.

Methanolysis of the Polysaccharide

The polysaccharide (1 g.) was heated under reflux with 3% methanolic hydrogen chloride (25 ml.) for six hours; 15% methanolic hydrogen chloride (5 ml.) was then added at 16-hr. intervals, four times in all, the total time of hydrolysis being thus 70 hours. The resultant solution was diluted with water and heated at 100° for six hours. After neutralisation with sodium hydroxide, chromatographic examination in solvent 3 failed to reveal the presence of glucuronic acid or its lactone.

Formic Acid Hydrolysis

The polysaccharide (1 g.) was heated with 98% formic acid (50 ml.) under nitrogen with stirring for seven hours. The neutral sugars were separated by elution through a column of IR-45-B resin (acetate form); the column was washed with water until the eluate did not give a positive Molisch reaction. The acid fraction was recovered by elution with N formic acid; after repeated extraction with ether, the aqueous acid fraction was evaporated to dryness and exhaustively extracted with ethanol. Chromatographic examination of the concentrated ethanolic extracts revealed a single spot with the same mobility

(chromatographically and ionophoretically) as the aldobiuronic acid described above. In a repeat of the experiment, spots corresponding to tri- and tetra-oligouronic acids were observed.

Large-scale Separation of the Acidic Fraction

The polysaccharide (5.95 g.) was hydrolysed with N sulphuric acid (7 hr., 600 ml.). The cooled solution was neutralised with barium hydroxide, together with a little barium carbonate. Hot-water extraction of the precipitated salts, together with the original filtrate, afforded 3.89 g.(66%) of hydrolysed material. The mixture of free sugars and barium uronates was applied to a cellulose column (4.2 x 36 cm.) and eluted with n-butanol ~~s~~-saturated with water. After separation of the neutral sugars, the column was washed with water (1 litre) and the aqueous eluant of barium uronates evaporated to small volume. Treatment with IR-120-H resin (4 g.) and filtration gave an acidic solution (pH 1-2), which was evaporated to dryness at 35°/15 mm. (1.57 g.).

The mixture of uronic acids was redissolved in water (10 ml.), mixed to a slurry with cellulose and freeze-dried. The resultant powder was applied to the top of a cellulose column (74 x 3.6 cm.), and eluted as before (page 34). 8-ml. fractions were collected at ten-minute intervals: $R_{Gl.}$ values are recorded for solvent 3.

Fraction	Tube	Wt.(mg.)	$R_{Gl.}$	Identity
1	1-310	144		Neutral sugars,etc..
2	311-450	227	1.05	Biuronic acid
3	451-550	58		Mixture
4	551-650	45	0.88	Trisaccharide
5	651-700	27		Mixture
6	701-800	51	0.63	Tetrasaccharide
7	water eluate	974		Higher oligosaccharides
		1526 mg. (98%)		

Each of the various fractions, after evaporation to small volume (10 ml.), was treated with barium hydroxide (to remove traces of sulphuric acid) and filtered. The filtrate was exhaustively treated with ether in a continuous extractor to remove final traces of acetic acid. Deionisation with IR-120-H resin, followed by evaporation to dryness, afforded the various fractions as clear acidic syrups.

Fraction 1 : The syrup consisted of glucose, xylose and rhamnose (paper chromatography). No evidence for the presence of glucuronic acid or its lactone was obtained, although all paper chromatograms exhibited streaking effects, possibly due to the presence of degradation products.

Fraction 2 : 227 mg. ; $R_{Gl.}$ 1.05.

The material was identical with the biuronic acid referred to earlier. A more detailed investigation of its properties is described on page 40.

Fraction 4 : 45 mg. ; $R_{Gl.}$ 0.88

$[\alpha]_D$ + 4°, (c. 0.5)

The syrup had molecular weight 508 g. (calc. for glucuronosyl-O-rhamnosyl-O-glucuronic acid, 514 g.); equivalent weight 249 g. (calc., 257 g.); $R_{G.A.}$ 0.68 (solvent 8). A portion was converted to the ester glycoside with 1% methanolic hydrogen chloride, and reduced as described in the case of the aldobiuronic acid. Chromatographic examination and estimation of the hydrolysed product gave :-

Glucose : rhamnose :: 1.76 : 1.

Fraction 6 : 51 mg. ; $R_{Gl.}$ 0.63

$[\alpha]_D$ + 6°, (c. 1.2)

The material had molecular weight 1050 g. (calc. for a tetrasaccharide, ca. 650 g.); equivalent weight 690 g. (calc. for a tetrasaccharide with 1 uronic residue, ca. 650 g.; with 2 residues, ca. 330 g.); $R_{G.A.}$ 0.44 (solvent 8). The syrup (22 mg.), treated as previously described, afforded 9 mg. of reduced hydrolysate. The relative molar proportions of the constituent sugars were :-

Glucose : rhamnose :: 1 : 0.83.

Fraction 7 : 974 mg. ; $R_{Gl.}$ 0 - 0.4

A sample of the material was hydrolysed (16 hr., 2 N HCl), and the neutralised, decationised product examined chromatographically (solvents 3 and 8). Spots corresponding to the acidic oligosaccharides described above were obtained, together with a faint trace of pink spots corresponding

to degradation products encountered in the neutral sugar separation. A sample (96 mg.) was esterified, reduced and hydrolysed in the usual manner. The resultant syrup (23 mg.) contained glucose (1 part), rhamnose (0.88 parts) and xylose (faint trace). It was therefore concluded that this fraction consisted almost entirely of unhydrolysed residues of rhamnose and glucuronic acid.

Examination of the Biuronic Acid

The syrup (227 mg.) had $[\alpha]_D$ - 6° (c. 0.5); $R_{Gl.}$ 1.05 (solvent 3); $R_{G.A.}$ 0.85 (solvent 4). It is henceforth referred to as the biuronic acid.

Determination of equivalent weight was carried out by titration against 0.0113 N sodium hydroxide solution (phenolphthalein indicator) :-

24.60 mg. of biuronic acid = 6.60 ml. of NaOH solution (0.0113 N)

i.e. E.W. = 328 g.

To determine reducing power, a weighed sample of the biuronic acid was treated with iodine solution (0.066 M - 2 ml.), together with excess of sodium hydroxide solution (0.175 N - 2 ml.). After standing for two hours, the mixture was acidified with sulphuric acid (0.25 N - 5 ml.), and the liberated iodine titrated against sodium thiosulphate (0.005 N), using starch indicator. The weight of rhamnose corresponding to the titre was then read off from a graph constructed by reacting known weights of rhamnose under the same conditions; comparison of the actual weight of the biuronic syrup with the theoretical weight of rhamnose afforded the required molecular weight

Rhamnose (monohydrate - mg.)	0	2.2	3.3	4.8	9.2	11.1
$Na_2S_2O_3$ (0.005N-ml.)	22.34	20.38	18.57	17.70	12.78	11.42
Titre difference	-	1.96	3.77	4.64	9.56	10.92

Biuronic acid (12.4 mg.) = 16.16 ml. of $Na_2S_2O_3$

Titre difference = 6.18 ml. = 6.55 mg. of rhamnose monohyd. (cf. graph)

$$\text{M.W. of biuronic acid} = \text{M.W. of rhamnose monohyd.} \times \frac{12.4}{6.55}$$

$$= 344$$

Chromatography of the Biuronic Acid

The syrup had R_{Gl} 1.07 (solvent 3); co-spotting with ammonium hydroxide gave a second distinct spot of R_{Gl} 0.57. This is comparable to the behaviour of D-glucurone, which normally runs as a single spot of R_{Gl} 2.4, but on co-spotting with ammonium hydroxide gives a second spot at R_{Gl} 1.00, corresponding to the free acid.

The biuronic acid had M_G 0.75 in borate buffer (pH 10); in acetate buffer (pH 5.5) run under the same conditions, it gave a single spot which diffused backward for ca. 2 cm. by electroendosmosis; glucose and glucurone behaved similarly. When glucurone and the biuronic acid were treated with ammonium hydroxide prior to running in acetate buffer, each gave a second spot migrating for ca. 15 cm. In this case, the behaviour of the second spot is comparable to that of galacturonic acid, which moved 14 cm. when subjected to ionophoresis in acetate buffer.

All attempts at hydrolysis or

methanolysis of the biuronic acid, over varying periods of time, invariably gave unchanged material or degradation products of the furfural type. No evidence for either rhamnose or glucuronic acid could be obtained (paper chromatography).

Reduction of the Biuronic Acid

The biuronic acid (204 mg.) was converted to the ester glycoside (210 mg.), and reduced with lithium aluminium hydride as previously described. After filtration of aluminium hydroxide, the mixture was heated at 60° to drive off the organic liquid, and the remaining aqueous solution made up to 40 ml. A portion of this solution (4 ml.) was deionised with IR-4-B and IR-120-H resin, and evaporated to a clear glass (14.2 mg.).

This material was treated with sodium periodate solution (0.015 M, 10 ml.), and the uptake measured by the spectrophotometric method (58) (cf. page 55.). After 44 hours, uptake was equivalent to 2.8 moles of periodate per mole of disaccharide glycoside, and only slowly proceeded beyond this figure.

The remaining solution of disaccharide glycoside (128 mg., 36 ml.) was treated with an equal volume of 50% sodium hydroxide in an atmosphere of nitrogen, at 5°, and dimethyl sulphate (17 ml.) added dropwise with cooling over six hours. The mixture was allowed to stand overnight and then treated with a further quantity of methylating agents as

before. Following a third methylation, the mixture was neutralised by addition of sulphuric acid, and filtered free from precipitated sodium sulphate. The remaining solution was exhaustively extracted with chloroform; evaporation of the organic extract afforded the reduced methylated disaccharide glycoside (98 mg.).

The syrup ($[\alpha]_D$ + 7° (c. 2.0, methanol)) was hydrolysed for seven hours in N hydrochloric acid containing 50% methanol. Neutralisation with silver carbonate, followed by chloroform extraction of the residue, afforded the hydrolysed disaccharide as a clear syrup. Chromatography (solvent 6) indicated the presence of tetra-O-methylglucose, di-O-methylrhamnose, and a trace of mono-O-methylrhamnose. The hydrolysed material (84 mg.) was applied to a sheet of thick paper (40 x 40 cm.) and eluted overnight in solvent 7. After location of the separated sugars by spraying of guide-strips, the portions of paper containing these materials was exhaustively extracted with water and with ethanol. Evaporation to dryness, followed by dissolution in water and filtration through hard filter-paper, finally afforded :-

1/ - Chromatographically pure 2:3:4:6-tetra-O-methylglucose;(36 mg.); m.p. and mixed m.p. 94°; $[\alpha]_D$ + 79°(c. 0.9); (Found: OMe 51.9, Calc. for tetra-O-methylhexose: OMe 52.5%).

2/ - Di-O-methylrhamnose (34 mg.); $[\alpha]_D$ + 42° (c. 2.9).

Chromatography and ionophoresis indicated that the syrup was

identical with 2:3-di-O-methylrhamnose (cf. page 55). Oxidation with periodate and chromatographic examination of the product (59) in solvent 6 gave one main spot at R_F 0.81; spots at R_F 0.72, 0.78 and 0.85 were obtained from the 2:4-isomer. Ionophoresis (borate buffer, pH 10) gave a single spot which diffused back from the starting-line; the 3:4-isomer has M_G 0.36.

It is considered therefore, from all the evidence available, that the aldobiuronic acid is 4-β-D-glucuronosylrhamnose.

Attempted Isolation of Neutral Oligosaccharides

After an initial small-scale experiment had shown the feasibility of the procedure, a large-scale partial hydrolysis of the polysaccharide was carried out. The polysaccharide (10 g.) was heated at 100° for 30 minutes with 0.3 N sulphuric acid (100 ml.). Ethanol (9 volumes) was then added to the stirred solution; precipitated material was removed by centrifugation and rehydrolysed as before, the procedure being repeated nine times in all.

The supernatant alcohol solutions were combined and evaporated to small volume (50 ml.), care being taken to ensure that all the alcohol had been removed from the solution. The resultant mixture of mono- and oligo-saccharides was adsorbed on a charcoal column (30 x 5 cm.) (60) and eluted with water (2 litre) until the eluate was no longer acidic. Evaporation of the aqueous acid eluate to small volume was followed by neutralisation with barium carbonate and aqueous

extraction of the residue; evaporation afforded a syrup (2.5 g.) which consisted of neutral monosaccharides.

The column was then eluted with 20% ethanol (3 litres) until no positive reaction for carbohydrate was obtained from the eluate. The solution was evaporated to small volume, and the free uronic acids neutralised by addition of barium carbonate. Evaporation to dryness afforded a white solid (1.3 g.); paper chromatography in basic eluants (solvents 1 and 2) indicated a mixture of neutral oligosaccharides, together with barium uronates and a trace of monosaccharides.

Paper chromatography in solvent 6 gave very little separation after 48 hours; prolonged elution (48 hours) in solvent 2 resulted in good separation between the various oligosaccharides. However, attempted separation on a cellulose column, using this eluant, gave only a gradation of mixed fractions. The separated fractions were therefore recombined and eluted on thick paper (solvent 1, 3 days). Although separation was more marked than in the case of the cellulose column, in no instance was a chromatographically pure oligosaccharide obtained.

Acetylation of the Polysaccharide

The polysaccharide (13.8 g.) was dispersed in an Ato-mix with dried pyridine (700 ml.) and formamide (100 ml.). After addition of acetic anhydride (200 ml.), the mixture was shaken over three days; the resultant brown solution was then added with cooling to an equal volume

of water. Following dialysis and evaporation to small volume, the acetylated polysaccharide was obtained on freeze-drying as a white powder(15.6 g.; Found: acetyl, 13.6%).

The material was reacetylated as before, yielding 14.5 g. of product (Found: acetyl, 22.4%). After a third acetylation, the off-white product had an acetyl content of 22.3% (12.84 g.).

Fractionation of the Acetylated Polysaccharide

The acetylated material (12.7 g.) was exhaustively extracted with chloroform in a Sohxlet apparatus. Evaporation of the chloroform extracts to small volume, followed by addition of light petroleum, afforded soluble polysaccharide (A) as a white powder (0.91 g.). The remainder of the original material (chloroform-insoluble acetate) (11.5 g.) will be referred to henceforth as polysaccharide (B).

Properties of Polysaccharide (A)

The acetylated material had $[\alpha]_D$ + 71° (c., 1.2, chloroform)(Found: sulphate, nil.). Deacetylation of a portion with sodium methoxide afforded a polysaccharide (70 mg., 50% yield), which gave a purple colour with iodine; this property was destroyed by prior treatment with α-amylase (61). Hydrolysis of a sample (20 mg.) and estimation of the sugars present (52) gave the following molar proportions :-

Glucose		Mannose		Xylose
1.00	:	0.31	:	0.17

Deacetylation and Methylation of Polysaccharide (A)

The acetylated material (0.82 g.) was dissolved in 30% sodium hydroxide solution (20 ml.) and dimethyl sulphate (9 ml.) was added dropwise with stirring over six hours. The ice-cooled reaction was conducted throughout in an atmosphere of nitrogen. After standing overnight, the mixture was treated with a further quantity of methylating agents as before. (63).

The mixture was then brought to pH 7 and dialysed to remove inorganic matter. Thallium hydroxide (2 g.) was added, and the mixture freeze-dried. The white powder was refluxed with methyl iodide (10 ml.) until an alkaline reaction was no longer obtained (16 hr.) (64). Following evaporation of the methyl iodide, the yellowish residues were extracted in the cold with methanol (3 x 25 ml.), and with 50% aqueous methanol (3 x 25 ml.). Hot extractions were carried out with 50% aqueous methanol (3 x 25 ml.), and with water (3 x 25 ml.). The various extracts were combined, evaporated to small volume, and treated with thallium hydroxide and methyl iodide as before. After a third methylation the methyl iodide was evaporated off, and the residues exhaustively extracted with chloroform; evaporation afforded methylated polysaccharide as a pale yellow syrup (0.34 g.).

The material was refluxed in methyl iodide (30 ml.), and silver oxide added gradually over six hours (5 g.). Following filtration and evaporation, the syrup was again methylated with Purdie reagents (65). After

filtration and exhaustive chloroform extraction of the combined silver residues, evaporation yielded 0.324 g. of polysaccharide, with $[\alpha]_D$ + 42° (c. 1.03, chloroform) (Found: OMe 38.6%).

The polysaccharide (0.30 g.) was heated under reflux with 4% methanolic hydrogen chloride (20 ml.) for 3½ hr. An equal volume of N hydrochloric acid was then added, and hydrolysis continued for a further 3½ hr. The solution was neutralised by addition of silver carbonate, and the filtrate evaporated to dryness. Exhaustive chloroform extraction of the combined residues, followed by dissolution in a minimum of 50% methanol and reprecipitation with hydrogen sulphide, finally afforded methylated hydrolysate as a yellow syrup (0.28 g.).

Investigation of the Methylated Sugars (A)

The mixture of methylated sugars (0.24 g.) was dissolved in a minimum of ethanol and spotted on to several discs of filter-paper. These were packed on a cellulose column (1.9 x 45 cm.) and cellulose (1 cm.) added on top. Elution was effected with a water-saturated mixture of light petroleum - n-butanol (7:3), 7 ml. fractions being collected at 15 min. intervals. The column was finally eluted with water, the eluate being added to those fractions in which no trace of sugars had been found. R_G values are recorded for solvent 6.

Fraction 1 : 1-71 ml. ; 21.1 mg.

R_G 1.00 ; $[\alpha]_D$ + 73° (c. 0.24)

(Found: OMe, 51.1; Calc. for tetra-O-methylhexose, 52.5%). The syrup gave only glucose on demethylation (paper chromatography), and was chromatographically identical with 2:3:4:6-tetra-O-methylglucose. Identity was proved by formation of the crystalline anilide, with m.p. and mixed m.p. 115°.

Fraction 2 : 72-189 ml. ; 11.2 mg.

R_G 1.00, 0.91, 0.81

Chromatographic comparison against standards indicated a mixture of 2:3:4:6-tetra- and 2:3:6-tri-O-methylglucose, and 2:3:4-tri-O-methylxylose. Demethylation gave spots corresponding to glucose and xylose, with possibly a faint trace of mannose.

Fraction 3 : 190-364 ml. ; 83.6 mg.

R_G 0.81 ; $[\alpha]_D$ + 72° (c. 0.78)

(Found: OMe, 40.3; Calc. for tri-O-methylhexose, 41.9%). the syrup was chromatographically identical with 2:3:6-tri-O-methylglucose (solvents 6 and 7), and gave only glucose on demethylation. An aliquot was allowed to stand at room temperature in 0.1 N methanolic hydrogen chloride (c. 1.12), and the change in rotation observed :-

Hours	0.2	1	4	5	21	29	45
α_D	+74	+52	+26	+24	-19	-25	-32 °

The final $[\alpha]_D$ was thus - 29°. This change in rotation is characteristic of 2:3:6-tri-O-methylglucose, indicating that the

-OH group on C_4 is free to form the methylglucofuranoside (66). Hydrolysis with 0.5 N Hydrochloric acid gave back the parent sugar. Attempts to form the di-p-nitrobenzoate on this material were unsuccessful.

The remaining portion of the tri-O-methylglucose was oxidised with bromine until a reducing action was no longer obtained (48 hr.). Removal of the bromine by aeration, followed by evaporation to dryness, afforded the lactone derivative as a clear syrup with $[\alpha]_D$ + 33°; attempts to form the crystalline phenylhydrazide on this material were unsuccessful.

Fraction 4 : 379-469 ml. ; 16.4 mg.

R_G 0.76 ; $[\alpha]_D$ + 9° (c. 0.7)

The syrup gave only xylose on demethylation. It was chromatographically identical with authentic 2:3-di-O-methylxylose, as distinct from the 2:4-isomer (R_G 0.69); ionophoresis against both these standards indicated identity with the 2:3-isomer, which is slightly more mobile than the 2:4 derivative.

Fraction 5 : 470-558 ml. ; 18.9 mg.

R_G 0.76 ; $[\alpha]_D$ + 16° (c. 1.0, methanol).

The syrup gave mannose and xylose on demethylation, and was evidently a mixture of fractions 4 and 6 (paper chromatography).

Fraction 6 : 589-1036 ml. ; 16.1 mg.

R_G 0.76 ; $[\alpha]_D$ + 16° (c. 0.8, methanol)

The syrup gave only mannose on demethylation; it was chromatographically identical with 2:3:6-, as distinct from 2:4:6- and 3:4:6-tri-O-methylmannose, when these substances were run as controls (solvent 6). Rotations recorded for the 2:3:6-isomer are usually around - 10° (67).

Fraction 7 : 1037-1365 ml.; 27.3 mg.

R_G 0.56 ; $[\alpha]_D$ + 52° (c. 0.9, acetone)

(Found: OMe, 29.2; Calc. for di-O-methylhexose, OMe, 29.6%). The syrup gave only glucose on demethylation, and was chromatographically identical with the 2:3-isomer (solvents 6 and 7). Identity as 2:3-di-O-methylglucose was proved by formation of the crystalline anilide, with m.p. 133°, undepressed by admixture with an authentic specimen kindly supplied by Prof. M. Stacey.

Fraction 8 : Water eluate ; 12.3 mg.

R_G 0-0.15 ; $[\alpha]_D$ - 9° (c. 0.3)

The relative immobility of this material, together with its purple colour under U.V. light, led to the inference that it consisted of the barium salts of uronic acids. Deionisation (IR-120-H resin) gave an acidic syrup, relatively immobile in neutral solvent (6); when eluted in an acidic solvent (5), the following series of spots were obtained :-

R_F :	0.70	0.45	0.40	0.34	0.15	0.08
Colour :	pink	pink	yellow	yellow	brown	yellow

Properties of Polysaccharide (B)

The acetylated polysaccharide had $[\alpha]_D$ - 38° (c. 1.15, pyridine); (Found: sulphate, 5.7; acetyl 22.0%). Hydrolysis of a sample and colorimetric estimation of the neutral sugars present gave the following molar proportions :-

Galactose	:	Glucose	:	Xylose	:	Rhamnose
0.06	:	0.56	:	1.69	:	1.31 .

Deacetylation and Methylation of Polysaccharide (B)

The acetylated polysaccharide (11.3 g.) was methylated twice with sodium hydroxide and dimethyl sulphate as in the case of polysaccharide (A); three thallium methylations were likewise carried out. After the third thallium methylation, the residues from all stages were combined and exhaustively extracted with chloroform. Evaporation yielded a yellow-brown syrup (5.4 g.; Found; OMe 34.7%). The material was subjected to four Purdie methylations, the last two failing to increase the methoxyl content. The fully-methylated polysaccharide (5.22 g.) had $[\alpha]_D$ - 28° (c. 1.3, chloroform); (Found: OMe, 35.1; sulphate 5.4%).

Attempted Fractionation of the Methylated Polysaccharide (B)

The methylated material (5.12 g.) was stirred twice with light petroleum (200 ml.), the insoluble portion being removed by centrifugation. Evaporation of the supernatant liquid afforded soluble polysaccharide as a white powder. The procedure was repeated with increasing proportions of chloroform to light petroleum in the solvent, until finally all the polysaccharide was soluble in a mixture containing 40% of

chloroform.

Fraction	1	2	3	4	5	6	7	8	Total weight (mg.)
Solvent (% chloroform)	0	5	10	15	20	25	30	40	
Weight (mg.)	24	47	106	386	635	972	943	2060	5,173

A small sample of each of the separate fractions was hydrolysed and examined by paper chromatography; each of the hydrolysates contained methylated uronic acids, and exhibited no appreciable quantitative difference in the neutral sugars present. It was therefore concluded that no fractionation had been achieved, and all of the fractions were recombined.

Hydrolysis of the Methylated Polysaccharide (B)

The methylated polysaccharide (5.1 g.) was hydrolysed as in the case of polysaccharide (A); the hydrolysate was neutralised by addition of silver carbonate and evaporated to small volume. After treatment with hydrogen sulphide to remove silver ions, the acid filtrate was brought to pH 7 by addition of barium hydroxide and a little barium carbonate.

Chromatography, together with the observation that the polysaccharide hydrolysate was fully soluble in ethanol but not in water, led to the view that the material was incompletely hydrolysed. Therefore a small portion was again hydrolysed for 28 hours, samples being neutralised at intervals of seven hours. On the basis of chromatographic comparison of these hydrolysates, it was decided that the most satisfactory time of

hydrolysis was 21 hours in all, extension of this time causing degradation.

The residual partially-hydrolysed methylated polysaccharide was redissolved in 4% methanolic hydrogen chloride (300 ml.) containing a little water, and heated under reflux for 14 hours. Neutralisation with silver carbonate, followed by treatment as described above, finally afforded the neutral hydrolysate as a white solid (4.1 g.).

Investigation of the Methylated Sugars (B)

The hydrolysed polysaccharide (4.05 g. was dissolved in a minimum of water and applied to several discs of filter-paper; these were then placed on top of a cellulose column (52 x 3 cm.), together with a little cellulose powder, and eluted as for polysaccharide (A). R_G values are quoted for solvent 6.

Fraction 1 : 87 mg. ; R_G 0.95

$[\alpha]_D$ + 22° (c. 0.8)

(Found: OMe 48.0; Calc. for tri-O-methylpentose, 48.4%). Demethylation afforded xylose, with a faint trace of glucose. The syrup was chromatographically identical with 2:3:4-tri-O-methylxylose, identity being proved by formation of the anilide derivative, with m.p. and mixed m.p. 97°.

Fraction 2 : 230 mg. ; R_G 0.86

$[\alpha]_D$ + 42° (c. 1.5)

(Found; OMe 32.4; Calc. for di-O-

methyldeoxyhexose, 32.3%). Rhamnose was the only sugar given on demethylation; the material crystallised slowly from methanol, affording crystals with m.p. 99°. Ionophoresis in borate buffer (pH 10) gave a single spot which diffused back from the starting-line, thus distinguishing from the 3:4-isomer, which has M_G 0.36. Comparison of the X-ray powder photograph of the crystalline sugar with that of authentic 2:4-di-O-methylrhamnose (m.p. 88°) proved the essential dissimilarity between the two materials. It was therefore concluded that the material from polysaccharide (B) was the 2:3-isomer, not hitherto reported crystalline (cf. $[\alpha]_D$ + 34°), (68).

Periodate Uptake of the Di-O-methylrhamnoses

Samples of 2:3- (3.00 mg.) and 2:4- (2.93 mg.) di-O-methylrhamnose were dissolved in a solution of sodium periodate (2 ml., 0.015 M). Samples (0.1 ml.) were extracted at the indicated intervals, and diluted to 25 ml. The absorption intensity of the resultant solution was then measured at 243 mμ in a Unicam U.V. spectrophotometer (58). After oxidation had reached a steady state, the reading was compared against a graph of absorbance intensities between 100 and 0% of periodate concentration. The resultant fraction of periodate consumed by the sugar solutions was then related to the total amount of periodate originally present, and thus to the molar consumption ratios.

Blanks:

	E
0.015 M $NaIO_4$ - diluted to 1/250	0.59
0.015 M $NaIO_3$ - " " "	0.12

Time (hr.)	0.5	4	20	44
2:3-isomer	0.27	0.26	0.20	0.19
2:4-isomer	0.49	0.40	0.37	0.37

After 44 hr.,	% of $NaIO_4$ consumed	Molar consumption
2:3-isomer	85%	1.6 M
2:4-isomer	47%	0.9 M

According to the simple theory of α-glycol oxidation, the respective periodate uptakes would be expected to be 1 mole (2:3-isomer) and 0 moles (2:4-isomer) (70).

The solutions remaining after oxidation were spotted on a paper chromatogram (after addition of glycol) and eluted in solvent 6 (59). Spraying with aniline oxalate indicated one main spot at R_F 0.81 in the case of the 2:3-isomer; spots at R_F 0.72, 0.78 and 0.85 were obtained from the 2:4-derivative.

Fraction 3 : 35 mg. ; R_G 0.81

$[\alpha]_D$ + 70° (c. 0.95)

(Found: OMe 42.0; Calc. for tri-O-methylhexose, 41.9%). The crystalline material gave glucose on demethylation, and had m.p. 122°, undepressed by admixture with an authentic sample of 2:3:6-tri-O-methylglucose.

Fraction 4 : 250 mg.; R_G 0.77

$[\alpha]_D$ + 21° (c. 3.8)

(Found: OMe, 34.0%; Calc. for di-O-methylpentose, 34.8%). The syrup was chromatographically with 2:3- as distinct from 2:4-di-O-methylxylose, and xylose was the only sugar given on demethylation. The derived anilide had m.p. and mixed m.p. 125°.

Fraction 5 : 464 mg. ; R_G 0.60

$[\alpha]_D$ + 37° (c. 3.5)

(Found: OMe, 17.1; Calc. for mono-O-methyldeoxyhexose, 17.4%). The syrup gave only rhamnose on demethylation; ionophoresis in borate buffer (pH 10) gave a single spot with M_G 0.06, thus distinguishing from 3-O- and 4-O-methyl-rhamnose, with M_G 0.37 and 0.48 respectively.

Estimation of the periodate uptake of the derived methylglycoside, as described above for fraction 2, gave a consumption of 1.1 moles per mole of glycoside. Paper chromatography of the oxidation products of the free sugar gave one spot at R_F 0.78; 4-O-methylrhamnose on similar treatment gave one spot at R_F 0.95 (solvent 6).

The derived anilide had m.p. 146°; the melting-point was undepressed by admixture with an authentic specimen of m.p. 150° (kindly supplied by Dr. L. Hough).

Fraction 6 : 14 mg. ; R_G 0.52

(Found: OMe, 29.5; Calc. for di-O-methylhexose, 29.6%). The syrup gave only glucose on treatment

with hydrogen bromide, and was chromatographically identical with the 2:3-di-O-methyl sugar. Identity was proved by formation of the anilide derivative; nucleation with an authentic specimen (kindly supplied by Prof. M. Stacey) afforded crystalline 2:3-di-O-methylglucose anilide, with m.p. and mixed m.p. 132°.

Fraction 7 : 26 mg. ; R_G 0.30

This substance gave a pink spot corresponding to xylose on demethylation; its chromatographic and ionophoretic behaviour was distinct from any of the three mono-O-methylxylose isomers. The syrup had high mobility in acidic solvent (5, R_G 0.75), indicating that it was acidic in nature.

Investigation of the Methylated Acidic Hydrolysate

The water eluate from the column was evaporated to dryness, affording 2.06 g. of barium salts of partly-methylated acids. Deionisation (IR-120-H resin) afforded an acidic solution (pH 2-3); evaporation to dryness gave a clear syrup (1.61 g.). Chromatographic examination in acidic solvent (5) gave the following series of spots :-

R_F	0.72	0.53	0.33	0.25	0.13	0.10 - 0
R_G	0.87	0.66	0.41	0.30	0.16	0.12 - 0
Colour (an.ox.spray)	yellow	pink	brown	brown-pink	brown	brown-pink

Attempted separation on a cellulose column, using solvent 5 as eluant, resulted only in a gradation between the various sugars. The separate mixtures were recombined, and separated on thick paper (solvent 5), affording the following

Fractions	1	2	3	4	5	6
R_G	0.87	0.68	0.41	0.30	0.16	0.12
Weight (mg.)	161	105	154	117	360	207
$[\alpha]_D$	-12°	+12°	+4°	-9°	-4°	+6°
(c., water)	(1.6)	(1.4)	(3.1)	(2.3)	(3.6)	(4.1)

The R_G of tri-O-methylglucuronic acid in this solvent is 0.66; the substance has $[\alpha]_D$ + 58° (71).

Fraction 1 : The syrup (160 mg.) was converted to the ester glycoside (150 mg.); the material was then reduced with lithium aluminium hydride as in the case of the biuronic acid. After removal of the aluminium hydroxide, the combined filtrate and washings were evaporated to small volume (10 ml.). The solution was made normal with respect to hydrochloric acid and hydrolysed at 100° for four hours. Neutralisation (silver carbonate) and extraction of the residues with chloroform finally afforded the reduced material as a clear syrup (95 mg.).

Paper chromatography (solvent 6) revealed the presence of two main constituents (R_G 0.84, brown-pink; R_G 0.61, brown-yellow), together with a trace of substance with R_G 1.15. Separation on thick paper afforded the two main sugars:-

A/ - 2:3:4-tri-O-methylglucose (38 mg.); the substance was chromatographically identical with further quantities of the same sugar isolated from subsequent fractions.

B/ - 2-O-methylrhamnose (36 mg.); the syrup had R_G 0.61, $[\alpha]_D$ + 34° (c. 1.8). The syrup was chromatographically and ionophoretically identical with the mono-O-methylrhamnose from the neutral

fraction of polysaccharide (B); the products of periodate oxidation were similar in both cases, and different from those of the 4-O-methyl isomer.

Other Fractions : Fractions 2, 3, 4, 5 and 6 were similarly converted to the ester glycoside and hydrolysed after reduction :-

Fraction	2	3	4	5	6
Weight (mg.)	56	154	117	360	207
Yield (mg.)	31	92	68	204	82

Chromatographic examination of the reduced materials (solvent 6) revealed that fractions 2 - 5 each gave two spots, of R_G 1.15 and 0.84 respectively. Fraction 2 also contained a small amount of mono-O-methylrhamnose; fraction 6 gave a series of spots with the following R_G values :- 1.15 (brown), 0.84 (brown-pink), 0.61 (brown-yellow), 0.48 (pink), 0.33 (yellow), 0.18 (pink), 0.10 (brown).

Fractions 2 - 5 were combined and separated on thick paper, affording two fractions : -

A/ - 2:3:4-tri-O-methylglucose (142 mg.); the syrup (R_G 0.84) was chromatographically identical with an authentic sample of the same sugar. It had $[\alpha]_D$ + 53° (c. 0.66), (Found: OMe 41.2; Calc. for tri-O-methylhexose, 41.9%). Formation of the anilide, and recrystallisation from ether in the cold, afforded crystals with m.p. 131°; the melting-point was not depressed by admixture with an authentic sample of m.p. 139°. I.R. examination of the free sugar gave an identical spectrum with that of standard synthetic 2:3:4-tri-O-methylglucose.

B/ - A yellow non-reducing syrup (113 mg.); R_G 1.15 (solvent 6). After treatment with charcoal, the material had $[\alpha]_D$ + 41° (c. 2.0). Periodate uptake, as determined by the spectrophotometric method, gave a value of 1 mole per 143 g. of syrup. Measurement of formaldehyde release (69) gave a negligible value.

Reduction of 2-O-methylrhamnose

Small quantities (20 mg.) of both 2-O-methylrhamnose and its methylglycoside were treated with lithium aluminium hydride as in the case of the methylated acid fraction; hydrolysis of both materials revealed spots at R_G 1.15 (paper chromatography, U.V. light). The spot was considerably fainter in the case of the material which had been subjected to glycosidation, being accompanied by a large amount of mono-O-methylrhamnose. In the case of the non-glycosided, reduced material and the syrup from the acid fraction, comparison of their I.R. spectra revealed that while not exactly identical in the fingerprint region, the molecular structure of both substances was undoubtedly similar.

DISCUSSION

DISCUSSION

Before extraction of the water-soluble polysaccharide, organic-soluble colouring-matter, free sugars, etc., were removed by exhaustive treatment with 85% ethanol under reflux. This procedure was found of use hitherto in similar preliminary treatment of the grasses (72). In no case did treatment with ethanol remove all the green colouring matter from Acrosiphonia centralis; but since the unextracted material was insoluble in aqueous solution, it did not seriously interfere with isolation of the polysaccharide.

Various methods of extraction were investigated, in order to obtain the highest yield of material, and also to determine whether any fractionation might thus be achieved. In no case was any appreciable difference between the various extracts observed, nor was a polysaccharide containing only a single sugar isolated. Varying amounts of neutral sugar were however obtained from the hydrolysates of extracts isolated by different procedures. All extractions were followed by dialysis of the dissolved polysaccharide, to remove inorganic material originating in either the extracting solvent, or in the polysaccharide itself.

While hot-water extraction afforded polysaccharide material in 4.5% yield, extraction with hot ammonium oxalate gave yields of up to 12%. Preliminary analysis of these two extracts confirmed their fundamental similarity; since the ammonium oxalate procedure gave the highest yield of material, it was decided that all structural investigations would be carried

out on this material.

The residual weed, both before and after treatment with ammonium oxalate, was hydrolysed with sulphuric acid by the Monier-Williams procedure for detection of cellulose (49). Chromatographic examination of the hydrolysate did not indicate a large preponderance of glucose, as might have been expected had cellulose been the main skeletal polysaccharide. Instead, glucose, xylose and rhamnose were found in equal proportions, together with indications of uronic acids. Since these are the constituent sugars of the water-soluble polysaccharide, it appears that this polysaccharide forms the main skeletal material of the weed, and that cellulose, if present, is there in much smaller proportion than in land plants. Algal cellulose has been isolated from the residues of several brown weeds (17); on the other hand, the results of Preston et al. indicate that in many of the Chlorophyceae, cellulose, if present, does not form a major portion of the cell wall (18). The results on the weed under investigation, Acrosiphonia centralis, which was not examined by these workers, are in keeping with this view.

General Properties of the Polysaccharide

The polysaccharide, $[\alpha]_D$ - 31°, was isolated mainly as the ammonium salt, 3.6% of nitrogen being removed on distillation. The material was contaminated with about 3.5% protein, and had ash 10% and sulphate 7.8%. The sulphate in the ash (32%) comprised half the total sulphate - evidence that the sulphate residues are ethereally linked to the polysaccharide.

Ethereal sulphate has been widely found as an integral part of algal polysaccharides. It ranges from less than 1% in certain specimens of agar (22) to 33% in fucoidin (37). The sulphate in Acrosiphonia extract corresponds to about one sulphate residue to every eight sugar units. It is comparable to the water-soluble extract of the green weed, Caulerpa filiformis (20) and is in contrast to the extracts from Cladophora rupestris (46), and Ulva lactuca (47), both containing ca. 20% sulphate.

The uronic anhydride content (ca. 20%) corresponds to about one uronic acid group per four sugar units. The presence of such a high proportion of acid residues makes the polysaccharide exceedingly difficult to hydrolyse, with the added complication that degradation occurs on severe hydrolysis. Partial hydrolysis afforded a neutralised hydrolysate of which 45-50% consisted of the barium salts of acidic oligosaccharides. Later investigations provided evidence that these substances contained equimolar proportions of rhamnose and uronic acid units. A hydrolysate with 20% of uronic acid present as unhydrolysed barium uronosyl rhamnose would contain 16% of rhamnose and 6% of barium; i.e. 40-45% of the neutralised hydrolysate would consist of barium uronates, in reasonable agreement with the experimental findings. The uronic anhydride content of Acrosiphonia is in marked contrast to that of Cladophora (less than 5%), but similar to that of Ulva (ca. 20%).

The free-acid polysaccharide had an equivalent weight of 459 g. A polysaccharide with uronic anhydride

content of 20% would have an equivalent weight of 870 g., assuming the absence of sulphate and ash. The neutral polysaccharide had a sulphate content of 7.8%, which corresponds to 67 g. of sulphate (i.e. 0.70 equivalents) in 870 g.. Hence 870 g. corresponds not to 1 but to 1.70 equivalents; and the equivalent weight of a polysaccharide containing 20% uronic anhydride and 7.8% sulphate = 870/1.70 = 512 g. In view of the fact that the analyses of uronic anhydride and sulphate were on neutral polysaccharide, and that this contained ca. 10% of ash, it is permissible to allow 10% in the calculation of theoretical equivalent weight. The final calculated value then becomes 462 g., in good agreement with the experimental value.

Cetyltrimethylammonium bromide has been used to precipitate acidic polysaccharides in the form of their quaternary ammonium salts (73); it has been found that this procedure is effective in separating acidic from neutral polysaccharides, which are not affected by treatment with this reagent. Despite several attempts, no fractionation of A. centralis extract into polysaccharides with appreciably different neutral sugar content could be achieved; on one occasion analysis of both soluble and insoluble fractions revealed that both had a comparable sulphate content. Attempted fractionation with copper salts (74) was likewise unsuccessful, and often resulted in low yield of the polysaccharide.

The polysaccharide consumed one mole of periodate for every 120 g., accompanied by release of formic acid equivalent to one mole per 250 g. Oxopolysaccharide was

isolated in 68% yield after dialysis; hydrolysis of this material and analysis of the neutralised hydrolysate indicated the presence of small quantities of rhamnose and xylose. The consumption of periodate is equivalent to a little over one mole per anhydro-sugar residue, and indicates that most of the units in the macromolecule possess two free contiguous hydroxyl groupings. The formic acid release indicates the presence of a large number of units with three contiguous hydroxyl groups, although uncertainty as to the nature of reaction with sulphate groupings render observations on acid release less definite. The presence of xylose and rhamnose in small amount in the oxopolysaccharide may be due to the presence of 1:3 links, or might indicate branching at these residues. It will be seen later that methylation results in general support these conclusions.

Hydrolysis of the polysaccharide was followed by observation of rotational changes and by iodometric titration. After about 6-8 hours the iodine values became erratic, the rotational values became constant, and the solution darkened. Chromatography on samples extracted at intervals indicated that glucose, xylose and rhamnose were released within the first hour, accompanied by neutral oligosaccharides and traces of mannose and galactose. All traces of oligosaccharides having disappeared after about seven hours, it was decided, in conjunction with rotational and iodometric data, that hydrolysis should be carried out over this period; the presence of acidic oligosaccharides was apparent throughout the hydrolysis.

The molar proportions of neutral

sugars in the partial hydrolysate of the polysaccharide was determined by two different methods, the results being in substantial agreement. In general the colorimetric method, while it affords only relative estimates as distinct from absolute percentages, was found preferable in cases where a large number of analyses had to be carried out. Thus, for example, colorimetric estimation was made of the neutral sugars in sample hydrolysates of different extraction procedures. On the other hand, where an absolute comparison of the actual amounts of sugars present was desirable, the method of periodate oxidation and estimation of resultant formic acid was found more suitable. Analysis of the standard polysaccharide hydrolysed under normal conditions (7 hr., N H_2SO_4) gave the following results :-

Sugar	Galactose	Glucose	Xylose	Mannose	Rhamnose
%	1.2	10.2	17.6	2.1	16.1
Relative moles	0.12	1.0	2.2	0.21	1.6

Each of these sugars was then separated and characterised by rotational data and formation of authentic crystalline derivatives. In keeping with the analyses, large-scale separation did not afford more than 40% of the hydrolysate as neutral sugars.

Galactose - Less than 1% of the sugar is present in the polysaccharide, whereas the extract from the botanically closely related Cladophora rupestris contains ca. 30% of galactose. It has also been reported in the hydrolysates of the green weeds Chaetomorpha

and Enteromorpha (18), and is the main constituent sugar of agar and carrageenin.

D-Glucose - The sugar occurs widely among the algae in both reserve and structural polysaccharides (cf. introduction). It has been reported in the hydrolysed extracts of several green weeds, and occurs in Cladophora and Ulva extracts in amounts of 0.5 and 8% respectively. Evidence discussed later indicates that in the present instance the glucose exists in the form of a separate non-sulphated α-1:4-linked polysaccharide.

D-Mannose occurs but rarely among the algal polysaccharides; the only investigation of an algal mannan is that carried out on an extract from Porphyra umbilicalis (75). As in the case of galactose, the sugar is present in only minor proportion in Acrosiphonia extract, and it is not possible to decide whether the material has any structural significance in the main polysaccharide.

D-Xylose occurs fairly often among the hydrolysed extracts of the red and green weeds; it is present in Cladophora and Ulva in fairly large amount (15 and 9%, as compared with 17.6% in the present instance). A pure xylan has recently been extracted from Caulerpa filiformis (20). Evidence from differential solubility extracts have indicated that xylans function as major cell-wall structural materials in several of the green algae (18).

L-Rhamnose is present in relatively large amount in the polysaccharides of several of the green seaweeds (e.g. Ulva extract has rhamnose 31%). In the present instance, it has been found that at least some of the rhamnose is linked with the uronic acid residues.

The Acid Fraction of the Polysaccharide

Neither methanolysis of the polysaccharide followed by hydrolysis, nor hydrolysis with different concentrations of mineral acid or 98% formic acid over varying periods, afforded a monouronic acid; invariably either biuronic acid or degradation products were isolated. Likewise, hydrolysis or methanolysis of the isolated biuronic acid did not afford either the monouronic acid or the sugar combined with it, but again gave degraded material.

After removal of cations the partial acid hydrolysate was separated by column chromatography, affording some neutral sugars (9%), together with di- (15%), tri- (4%), and tetra- (3%) saccharides. A polymeric fraction (65%) was also isolated; prolonged acid hydrolysis of this material (16 hr., 2 N acid) afforded the same mixture of oligosaccharides as before.

The biuronic acid ($[\alpha]_D$ - 6°) had equivalent weight of 328 g., the calculated weight for glucuronosyl-O-rhamnose being 340 g.. Similarly the determined molecular weight was 344 g., in good agreement with the calculated weight of 340 g.. Severe methanolysis and hydrolysis having failed to yield any indication of the constituent sugars, the biuronic acid (in the form of its ester glycoside) was reduced with lithium aluminium hydride and the product hydrolysed. Chromatography revealed the presence of equimolar proportions of glucose and rhamnose, the former being characterised enzymatically and the latter by formation of the authentic crystalline benzoylhydrazone. It is considered that the glucose arose from the reduction of glycosidically-linked glucuronic acid residues.

The glycoside of the biuronic ester consumed 2.8 moles of periodate per mole, indicating that the mode of linkage is through C_2 or C_4 of the rhamnose residue; methyl 2- or 4-glucuronosylrhamnoside requires 3 moles of periodate for complete oxidation, whereas methyl 3-glucuronosylrhamnoside consumes only 2 moles. Methylation and hydrolysis of the reduced aldobiuronic glycoside ester afforded equimolar proportions of crystalline 2:3:4:6-tetra-O-methylglucose and syrupy 2:3-di-O-methylrhamnose; the latter sugar was identified by rotational and analytical data, together with chromatography and ionophoresis against authentic specimens. The tetra-O-methylglucose having arisen from glycosidically-linked reduced glucuronic acid, therefore the biuronic acid has the constitution 4-β-glucuronosyl-rhamnose; the β-configuration is conferred on the material in view of the negative specific rotation.

The $R_{Gl.}$ values of the biuronic acid were high (ca.1.00) as compared with those of other known biuronic acids (e.g. $R_{Gl.}$ of 2-O-glucuronosylrhamnose = 0.24 (76)); the possibility of it existing as the lactone was thus suggested. The syrup, which had $R_{Gl.}$ = 1.07 (solvent 3), gave a second spot with $R_{Gl.}$ = 0.57 on treatment with ammonium hydroxide prior to elution. Comparison with D-glucurone, which also gives an acid and lactone spot on similar treatment, thus indicated that the biuronic acid as isolated was in the lactone form. Comparison with D-glucurone ionophoretically again indicated the similarity between the two materials; both the biuronic acid and the monosaccharide gave a single spot in borate buffer (M_G 0.75 and 0.80 respectively) while treatment with ammonium hydroxide prior

to elution gave a second spot in both instances. Ionophoresis in acetate buffer (pH 5.5) of the biuronic acid and glucurone gave single spots which diffused back from the starting-line, indicating the absence of charged groups at this pH. It was thus indicated that the biuronic acid does indeed exist in the lactone state; in this regard it is worth noting that the equivalent weight (328 g.) is closer to that calculated for a ~~lactone~~ lactone of glucuronosylrhamnose (322 g.) than the calculated weight for the free biuronic acid (340 g.).

The trisaccharide had equivalent and molecular weights of 249 and 508 g. respectively; a structure such as glucuronosyl-O-rhamnosyl-O-glucuronic acid, or di-glucuronosylrhamnose would require 257 and 514 g. respectively. In agreement with this theory, reduction and hydrolysis afforded glucose and rhamnose in the molar proportions of ca. 2 to 1. In view of the apparent stability of the glycosidic linkage to acid hydrolysis, isolation of a substance with a terminal uronic unit is not easily explicable, and it appears more likely that the trisaccharide has the structure diglucuronosylrhamnose.

The tetrasaccharide gave equimolar proportions of glucose and rhamnose after hydrolysis of the reduced material, thus indicating a structure composed of two combined biuronic residues. The equivalent and molecular weight determinations gave results of about half the theoretical value; this is perhaps ascribable to the difficulty of purification of the small amount of syrup available (50 mg.) from the cellulose column, although there was no such difficulty in the case of the

trisaccharide.

Reduction and hydrolysis of the polymeric fraction from the acid portion of the hydrolysate afforded equimolar proportions of glucose and rhamnose, together with a faint trace of xylose. The low yield (24%) from this reaction is attributable to the low solubility of the material in the organic solvents employed for reduction.

It was thus concluded that the acidic portion of the polysaccharide consisted almost entirely of rhamnose and glucuronic acid residues in equimolar proportions. As detailed earlier (page64), this structure is in agreement with the uronic anhydride content and with the proportion of barium uronates in the neutralised hydrolysate. Characterisation of the biuronic acid fraction has established that this material consists of glucuronic acid glycosidically linked to C_4 of the rhamnose residue; it is probable that the disaccharide exists in the lactone form in the free state at any rate. Isolation and analyses of other oligosaccharides indicated that acidic and neutral sugars were alternately linked throughout the acidic portion of the polysaccharide. Such a structure is in keeping with the extreme resistance to hydrolysis of the acidic material.

Fractionation of the Polysaccharide

Mild acetylation of the polysaccharide gave an acetylated material in which the acetyl content could not be raised above 22.3%. Chloroform extraction of this acetate afforded a non-sulphated, acid-free, glucose-rich acetate (A) with $[\alpha]_D$ + 71° (7.5% yield). Although approximately 50% of the

glucose in the original polysaccharide remained in the insoluble portion (B)($[\alpha]_D$ - 38°), and although later experiments (page 76) verified that in this material the glucose was linked together in the same way as in the soluble fraction, exhaustive extraction with chloroform failed to yield any more soluble polysaccharide. Fractionation by differential solubilities of the acetylated or methylated polysaccharide has also proved successful in the case of the sulphated extracts from Cladophora and from Ulva; in both cases a more soluble glucose-rich, low-sulphated fraction has been separated from the main bulk of the material.

Examination of the relative proportions of the sugars in the present instance gave : -

	Gal.	Gluc.	Mann.	Xyl.	Rhamn.
Polysaccharide(A):		1.00	0.15	0.27	—
Polysaccharide (B):	0.06	0.57	-	1.68	1.31

Now neutral sugars account for over 50% of the total polysaccharide, or approximately 50% of polysaccharide (B); they also account for almost 100% of polysaccharide (A). Therefore the relative molar proportions of the sugars in the original material should be obtained by adding the relative moles of sugars in (B) (x 0.5 to allow for the acidic portion) to the relative moles of sugars in (A) (x 0.076 to allow for the different weights of polysaccharide). As detailed in the following table, such calculations are in fair agreement with the experimental findings.

	Gluc.	Xyl.	Rham.
Rel. Moles (A)	1.00	0.27	-
Weights (gm.)	0.65	0.14	-
Rel. Moles (B)	0.57	1.68	1.31
Weights (g.)	1.01	2.42	2.10
Weight of A + B	1.66	2.56	2.10
Rel. Moles (A + B)	0.86	1.65	1.25
Found for Acetate (unfractionated)	1.00	1.65	1.30

Methylation of polysaccharide (A) gave rise to a product with 38.6% methoxyl value; hydrolysis and separation of the resultant mixture of methylated sugars yielded 2:3:4:6-tetra-, 2:3:6-tri-, and 2:3-di-O-methylglucose, in the relative proportions of 1 : 4 : 1. These substances were characterised by analytical data, together with the formation of crystalline derivatives. Because of the positive rotation of the polysaccharide, it is considered that the predominant linkage of these sugars is of the α-type; the comparatively low value ($[\alpha]_D$ + 42°) is possibly due to contamination by polysaccharide (B), with $[\alpha]_D$ - 28°.

Indications of the presence of di- and tri-O-methylxylose derivatives were also obtained, together with traces of acidic oligosaccharides; these substances all occur in the hydrolysate of methylated polysaccharide (B), and their occurrence in the present instance is probably due to incomplete fractionation of the two polysaccharides. It is not possible at present to decide whether the mannose present in the hydrolysate of polysaccharide (A) forms an integral part of the molecule.

Whereas the glucose-rich fraction separated from the water-soluble extract of Cladophora appeared to consist of β-1:3-linked residues, recent fractional extraction of this weed has yielded an α-1:4-linked glucan (77). The methylated glusose-rich fraction from Ulva on hydrolysis gave evidence for the presence of 2:3:6-tri- and 2:3-di-O-methylglucose; however the hydrolysate contained a large proportion of rhamnose derivatives, and it is impossible to decide if the authors had indeed separated a glucan.

Summarising then, the results indicate that the following linkages are present in the glucose-rich fraction from Acrosiphonia extract :-

Gl .....,	....^{4}Gl ....,	....^{4}Gl ....,	Xl...., ...^{4}Xl...,
(1 part),	(4 parts),	^{6} (1 part),	....^{4}Ml....; (25%).

Polysaccharide (B)

The acetylated polysaccharide (B) had an acetyl content of 22%, and a sulphate content of 5.7%. Methylation as for polysaccharide (A) afforded a product with methoxyl content of 34.7%. The methylated material proved very difficult to hydrolyse, 21 hours with N acid being required to effect even partial hydrolysis. As in the case of the original separation into neutral and acidic sugars, approximately 50% of the methylated hydrolysate was found to consist of neutral sugars.

2:3:4-Tri-O-methylxylose (1 part), 2:3-di-O-methylxylose (3 parts), 2:3-di-O-methylrhamnose (3 parts) and 2-O-methylrhamnose (5 parts) were obtained and characterised

by formation of authentic crystalline derivatives. Small amounts of 2:3:6-tri- and 2:3-di-O-methylglucose (in ratio 3:1) were also separated; the similarity of the nature and proportion of these linkages with those obtained from polysaccharide (A) suggests that the presence of glucose in the hydrolysate of polysaccharide (B) is due to incomplete fractionation.

The relatively large amount of mono-O-methylrhamnose is especially noteworthy, being far in excess of the quantity of rhamnose separated from the partial hydrolysate of the original polysaccharide. Since more drastic conditions were required to hydrolyse the methylated material, this extra rhamnose has probably arisen from units chemically linked in the acidic portion of the molecule to uronic acid residues; such material would not be liberated in the original hydrolysis.

Examination of the Acidic Fraction (B)

After the methylated neutral sugars had been separated by column chromatography, deionisation of the residual mixture of barium uronates afforded a syrup which gave a series of spots corresponding to partly-methylated oligouronic acids (paper chromatography). After attempted separation on a cellulose column had proved unsuccessful, several pure fractions were separated on thick paper.

The first fraction, after conversion to the ester glycoside, was reduced and hydrolysed. Equimolar proportions of 2:3:4-tri-O-methylglucose and 2-O-methylrhamnose

were separated from the hydrolysate. It appears very probable that this material arose from the same portion of the molecule as the 4-glucuronosylrhamnose isolated from the partial hydrolysate, and that it has the structure 2:3:4-tri-O-methyl-4-glucuronosyl-2-O-methylrhamnose; it is also indicated that the hydroxyl group on C_3 of the rhamnose was either joined to another sugar residue, or carried a sulphate grouping in the original polysaccharide.

Other separated acidic materials were similarly reduced and found to yield 2:3:4-tri-O-methylglucose on hydrolysis. The sugar was characterised by analytical data, and by the identity of its infra-red spectrum with that of an authentic synthetic specimen. In each case, the tri-O-methyl-glucose was accompanied by one other main component.

This unknown material has greater chromatographic mobility than tetra-O-methylglucose; the substance was non-reducing, and its periodate uptake was equivalent to one mole per 143 g. of syrup. These properties are in agreement with the structure 2-O-methyl-1:5-anhydro-rhamnitol, which would consume one mole of periodate per 149 g. It is difficult to see how this product could have arisen from the methylated acid fraction but it has been shown that lithium aluminium hydride reduction of rhamnose gives 1:5-anhydro-rhamnitol (78). With this possibility in mind, both 2-O-methylrhamnose and methyl 2-O-methylrhamnoside were each subjected to reduction with lithium aluminium hydride. A single spot, R_G 1.15 identical with the spot given by the reduced acid fractions, was obtained on chromatographic analysis of each of the reduced rhamnose derivatives, although the spot was somewhat

fainter in the case of the product from the glycoside. While it is only tentatively suggested that the material isolated from the reduced acid fractions is indeed 2-O-methyl-1:5-anhydro rhamnitol, and that during reduction fission of the glycosidic bonds occurred, it seems clear that the product arose from 2-O-methylrhamnose present in the acid fraction.

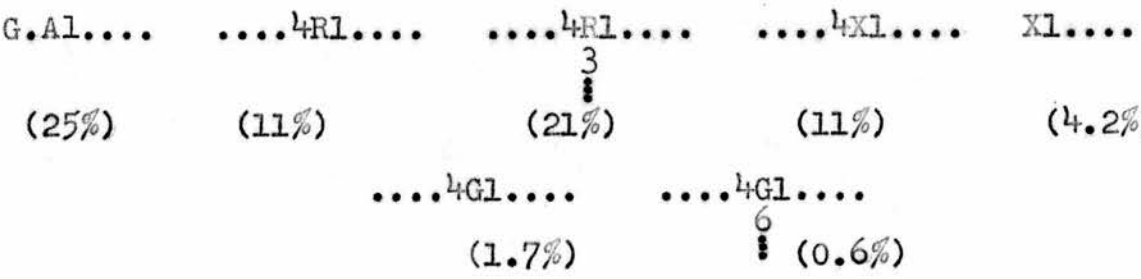
Reduction of the acid fraction with the smallest mobility (R_G 0.2) afforded a large number of spots. In view of the small amount of material available, this fraction was not further examined.

The sulphate content of the methylated polysaccharide (B) is in the same proportion as in the original extract, evidence that the sulphate groups are completely stable to alkali. Definite allocation of these sulphate groups in polysaccharide (B) is difficult. Their attachment to anhydro-glucose units can be ruled out in view of the separation of a sulphate-free glucan and of the small amount of glucose in the main polysaccharide material. Since the glucuronic acid residues are all fully-methylated, they must occur as glycosidically-linked end-groups and have no free hydroxyl groups to accomodate these residues. This leaves only xylose and/or rhamnose to be considered as points of linkage for the sulphate groupings. 2:3-di-O-methyl xylose and 2:3-di-O-methylrhamnose were isolated in approximately equal quantities, together with about twice as much mono-O-methyl-rhamnose. Any of these units could carry sulphate in the original polysaccharide, but it is considered probable that it is the rhamnose units that are sulphated. If, on the other hand, it

is C_4 of those xylose residues isolated as 2:3-di-O-methylxylose that is linked to sulphate, then practically all the xylose in the molecule is present as end-groups, many of which are sulphated. However, in spite of the failure to separate the extract into more than a single entity, it is considered probable that more than one individual polysaccharide is present, and that at least some of the xylose occurs as a separate xylan.

The isolation and characterisation of pure oligosaccharides from a partial hydrolysate would help to resolve this question. However it has not proved possible to separate any pure neutral oligosaccharides either on cellulose columns or on thick paper. Other workers in this field have experienced the same difficulty (78), and it is thought that the sulphate residues are in some way associated with this difficulty.

The results of methylation studies on polysaccharide (B) have revealed that the following linkages are present in the polysaccharide :-

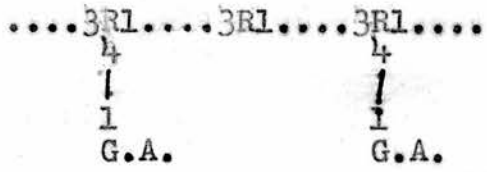


G.A. = glucuronic acid G = glucose R = rhamnose X = xylose

The rest of the molecule (ca. 24%) is made up of the non-reducing material isolated from the acid fraction, and originating, on the evidence so far available, from triply linked rhamnose.

No single structure can be advanced for

the main water-soluble polysaccharide material isolated from Acrosiphonia centralis on these results. It has however been established that at least part of the molecule is made up of a backbone of 1:3-linked rhamnose residues carrying glucuronic acid units as side-chains linked at C_4 :-



The structural significance of the other sugar residues has yet to be decided.

All these methylated sugars, with the exception of the mono-O-methylrhamnose, are derived from monosaccharide units which would be susceptible to periodate attack. This is in keeping with the periodate results, where an uptake of approximately one mole per sugar unit was found. The tri-O-methylxylose and tri-O-methylglucuronic acid have each originated from residues which would consume two moles of periodate per unit, but this is offset by the triply-linked rhamnose, which would be immune to periodate attack. The isolation of only a small quantity of rhamnose from the hydrolysate of the oxopolysaccharide is probably due to the resistance to hydrolysis of the acid portion of the polysaccharide.

The main fact which emerges from this investigation is that in spite of botanical resemblance, the metabolisms of Cladophora and Acrosiphonia are very different. Whereas the polysaccharide extract from Cladophora has a positive

rotation, and is built up mainly from arabinose, galactose and xylos units, that from Acrosiphonia is negative in rotation and consists of xylose, glucose, rhamnose and glucuronic acid residues. This latter polysaccharide more closely resembles the extract from Ulva lactuca; they both have comparable negative rotation and contain the same sugar residues. Moreover methylation studies gave end-group xylose, 1:4-linked xylose, 1:4-linked rhamnose and triply linked rhamnose as common to both materials. Beyond obtaining evidence for the presence of glucuronic acid, very little further work has been carried out on the acid fraction of Ulva; further investigation may well reveal closer similarity between these two green seaweeds.

Part 1 (b)

EXPERIMENTAL

Part 1 (b)

Extracts of Acrosiphonia centralis collected from Different Areas

A - From Weed supplied by Dr. Burrows, Liverpool

The dried weed (15 g.) was subjected to a preliminary treatment with 85% ethanol in a Sohxlet extractor during twelve hours. Hot-water extraction of the residual weed was followed by dialysis of the filtered solution. Evaporation to small volume, followed by addition of ethanol, afforded the polysaccharide as a white powder (0.7 g., 4.7%). The material had $[\alpha]_D$ - 20° (c. 0.4) (Found: $SO_4^{=}$, 19.6; Ash, 15.9; N 1%).

A sample was hydrolysed (16 hr., 2 N H_2SO_4) and the molar proportions of the sugars in the neutralised hydrolysate determined colorimetrically :-

Galactose	Glucose	Mannose	Arabinose	Xylose	Rhamnose
0.26	1.00	0.18	0.19	2.1	0.52 .

The neutral sugars in the hydrolysate were then separated from the barium uronates present by extraction with absolute ethanol. Deionisation of the residue gave an acidic syrup, which afforded a positive napthoresorcinol test for uronic acids. Paper chromatography in acidic solvents indicated the presence of an acidic material with the same chromatographic mobility as the biuronic acid described in Part 1 (a).

Acrosiphonia centralis supplied by Dr. de Valera (Galway)

After ethanolic extraction, the

dried weed (160 g.) was treated the same way as the previous sample. Freeze drying of the dialysed concentrated extract afforded 2.35 g. of polysaccharide material (1.7%) (Found: SO_4'', 5.5; Ash, 13.3; N, 3.1%; $[\alpha]_D$ - 45° (c. 1.0)).

Hydrolysis of a small sample and estimation of the relative sugar proportions gave :-

Galactose	Glucose	Arabinose	Xylose	Rhamnose
0.39	1.00	0.48	0.54	0.47

Acrosiphonia centralis collected at Nova Scotia (Dr. R. Lewin)

After removal of organic-soluble colouring matter, the dried weed (8 g.) was extracted with cold and with hot water, giving two polysaccharide materials. Comparative chromatography established that both extracts were identical in regard to the neutral sugar content of their hydrolysates. Hot and cold extracts were therefore recombined in solution, affording an off-white polysaccharide on freeze-drying (0.46 g., 5.5%). The material (Found: SO_4'', 19.6; Ash, 14.2%) had $[\alpha]_D$ - 100° (c. 0.3), together with uronic anhydride of 20% (mean of several determinations).

Hydrolysis of a small sample (50 mg., 2 N H_2SO_4, 16 hr.), followed by chromatographic examination of the neutralised hydrolysate, indicated the presence of glucose, xylose and rhamnose, together with trace quantities of mannose. Uronate salts were in evidence as oval spots of low chromatographic mobility. Estimation of the relative molar proportions of the neutral sugars was carried out by periodate oxidation (a) and by

colorimetry (b) :)

	Glucose	Mannose	Xylose	Rhamnose
(a)	1.00	0.18	0.83	1.35
(b)	1.00	0.16	0.77	1.20

Characterisation of Neutral Sugars

The polysaccharide hydrolysate (0.28 g. was applied to a cellulose column (40 x 2.7 cm.), and eluted with ½-saturated n-butanol; 7 ml. fractions were collected at 15 min. intervals, R_F values being quoted for solvent 1.

Fraction 1 : 2.35 mg. ; R_F 0.69

The syrup gave a positive Seliwanoff test, indicating that it was a decomposition product of the hydroxy-methyl-furfuraldehyde type.

Fraction 2 : 35.5 mg. ; R_F 0.55

$[\alpha]_D$ + 11° (c. 1.4)

The material crystallised from water in needles of m.p. 68°, undepressed by admixture with an authentic specimen of L-rhamnose hydrate.

Fraction 3 : 4.9 mg. ; R_F 0.48

The syrup, which gave a pink spot on spraying with aniline oxalate, was not chromatographically identical with any of the more common pentose sugars; positive reaction to a Seliwanoff test indicated that it was probably decomposition product.

Fraction 4 : 22.4 mg. ; R_F 0.44

$[\alpha]_D$ + 15° (c. 1.0)

The syrup crystallised in needles of m.p. 141°, undepressed by admixture with an authentic specimen of D-xylose. The material was further characterised by formation of the crystalline di-benzylidene dimethyl acetal derivative, of m.p. 208-10°.

Fraction 5 : 3.1 mg. ; R_F 0.38

The syrup was chromatographically identical with mannose (solvents 1 and 3). Attempts to prepare the crystalline phenylhydrazone derivative were unsuccessful.

Fraction 6 : 16.0 mg. ; R_F 0.33

$[\alpha]_D$ + 44° (c. 0.8)

The material was chromatographically identical with glucose, identity being conclusively proved by formation of the crystalline dichlorophenylhydrazone, of m.p. and mixed m.p. 153°. A small portion of the parent syrup was treated with the specific enzyme glucose oxidase; chromatographic examination of the deproteinised filtrate gave no evidence for the presence of the original hexose (cf. page 35).

Fraction 7 : 77.2 mg.; R_F 0.1

(Water eluate) - The solid (45%) of the total hydrolysate, consisted of the barium salts of a mixture of oligo-uronic acids. Deionisation (IR-120-H resin) afforded an acidic syrup; chromatography in acidic solvent (3) indicated the presence of one main constituent, R_{Gl} 1.05 (cf. biuronic acid, page 40).

DISCUSSION

After investigation of the water-soluble extract from Cladophora rupestris had been completed, it seemed appropriate that a similar investigation of the related weed, Acrosiphonia centralis (Spongomorpha arcta), should be carried out. Collection of pure samples of this weed is difficult, the first specimen (15 g.) being kindly supplied by Dr. R. Lewin of Nova Scotia. Preliminary analysis revealed that despite morphological similarity, the carbohydrate metabolism was very different from that of Cladophora rupestris. The complete absence of arabinose and galactose at once distinguished Acrosiphonia, together with comparatively high uronic acid content in the water-soluble extract.

It did not prove possible to find samples of the weed on the East coast of Scotland. Through the kindness of Dr. de Valera, Dr. Burrows, and Mr. Powell, samples were obtained from Isle of Aran (Ireland), Liverpool, and Millport (Isle of Cumbrae) respectively. The Millport weed was collected from a single area of small extent, which was completely free from other species of weeds. The water-soluble extract from this specimen differed from the Canadian sample in lower negative rotation and lower sulphate content, together with the presence of trace quantities of galactose. The Irish and Liverpool weeds on the other hand contained a small proportion of arabinose units, a higher proportion of galactose residues, and a lower proportion of rhamnose. It is impossible, until extracts of the weed at different stages of growth and from

different areas have been investigated, to decide whether these variations are due to seasonal changes, the growth of hybrids, or to the difficulty of collecting large quantities of pure species. It is worth noting in this respect that samples collected from Millport in the early stages of growth, at the height of the vegetative season, and just before the Autumn storms destroyed the beds, were each completely lacking in arabinose residues. In view of these difficulties, all the structural investigations reported in Part 1 of this thesis were carried out on Millport weed collected in May and June, 1957 and 1958.

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PART II

Structural Investigations Employing the Barry Degradation Technique

PART II

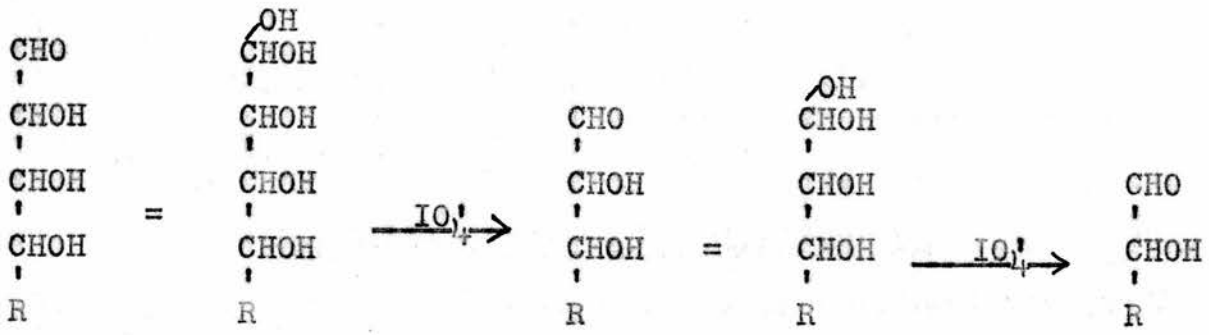
Periodate Oxidation and the Barry Degradation Procedure

Oxidising agents have found wide analytical and preparative use in carbohydrate chemistry (1). They are often of the halohydrin type, in which case the oxidative function results from reduction of the oxy-halogen complex. In this class may be placed oxidation by the periodate ion, IO_4^-, which has found extensive use because of its specificity, the quantitative nature of the reaction, and because oxidation can be readily conducted in aqueous solution.

While engaged in determining the relative proportions of periodic and iodic acid in solution, Malaprade observed the quantitative decomposition of a series of glycols to formaldehyde and formic acid (2):-

$$\begin{array}{l} CH_2OH \\ (CHOH)_n \\ CH_2OH \end{array} \xrightarrow{(n+1\ IO_4)} \begin{array}{l} H.CHO \\ + \\ n\ HCOOH \\ + \\ H.CHO \end{array} + (n+1)\ IO_3^- \qquad (n = 1 \text{ to } 4)$$

It remained for Fleury, in a series of periodate oxidation experiments on monosaccharides, to show that this reaction was apparently specific for 1:2-glycols; the secondary hydroxyl groups in the sugar chain were quantitatively converted to formic acid and the primary hydroxyl groups to formaldehyde(3). Oxidation of the aldehydic groupin reducing sugars was postulated to proceed through the transient formation of en hydrated form which was immediately attacked by periodate.

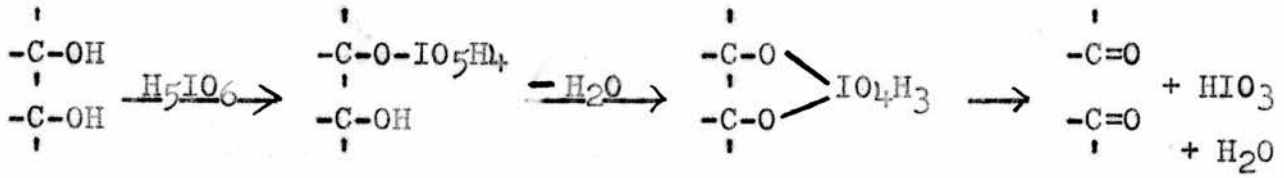


It has since become clear that the periodate ion will oxidise groupings other than 1:2-diols (cf. Part III, this thesis). α-hydroxy-aldehydes, 1:2-diketones, α-hydroxy-amines, and 1:2-diamines, are among the groupings which have been found to react with periodate at normal temperatures (4). At elevated temperatures, over a long period, the reagent apparently functions as a non-specific oxidant, breaking the C-C bond in such substances as acetone (5).

The oxidising action of the periodate ion is closely paralleled by that of lead tetraacetate (6). A notable difference is in their action on α-hydroxy acids, which are oxidised by the latter reagent in usual fashion, but only comparatively slowly by the former (7). The two oxidants also differ in their rate of attack at trans-glycol centres, which are oxidised by the periodate ion at a rate comparable to the cis-groups, but in general are attacked by lead tetraacetate much more slowly. Although periodate studies on sugar derivatives have been to some extent paralleled by lead tetraacetate oxidations, the former reagent has found wider use due to its suitability under aqueous conditions.

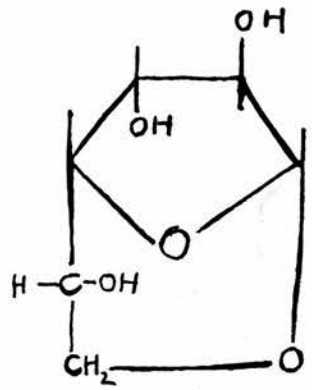
Although reaction by both oxidants

has been postulated to proceed by a free-radical mechanism (9), it is more generally held that fission of the C-C bond takes place through decomposition of a transient cyclic intermediate (10):-



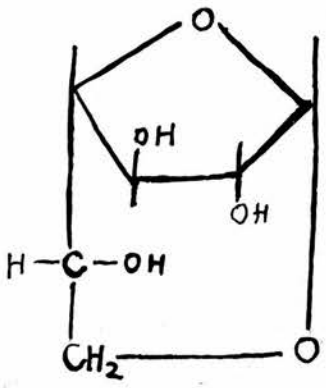
Proceeding on this hypothesis, atomic measurements and valency requirements, which would be neccessary for such a mechanism, were calculated and led to the successful prediction that sodium perbismuthate would oxidise in similar fashion (11). An examination of the kinetics of the IO_4^- - glycol reaction has led to the suggestion that co-ordination is neccessary to specificity, since it lowers the activation energy of the C-C bond to a level at which scission can easily take place (12).

Although absence of reaction with periodate has been taken as indicating the absence of 1:2-glycol groupings, several cases have been recorded where contiguous hydroxyl groups have failed to react. Such lack of reaction is attributed to steric factors which inhibit the formation of the co-ordination complex necessary for C-C fission. In the case of D-glucosan (1) and D-galactosan (11) immunity to periodate attack has been ascribed to the rigidity conferred by the double lactol ring system; any change in conformation of the sugar ring is thus prevented, and hence movement of the hydroxyl groups on C_2 and C_3 to a more favourable position for co-ordination.(13)



(1)

D-Glucosan



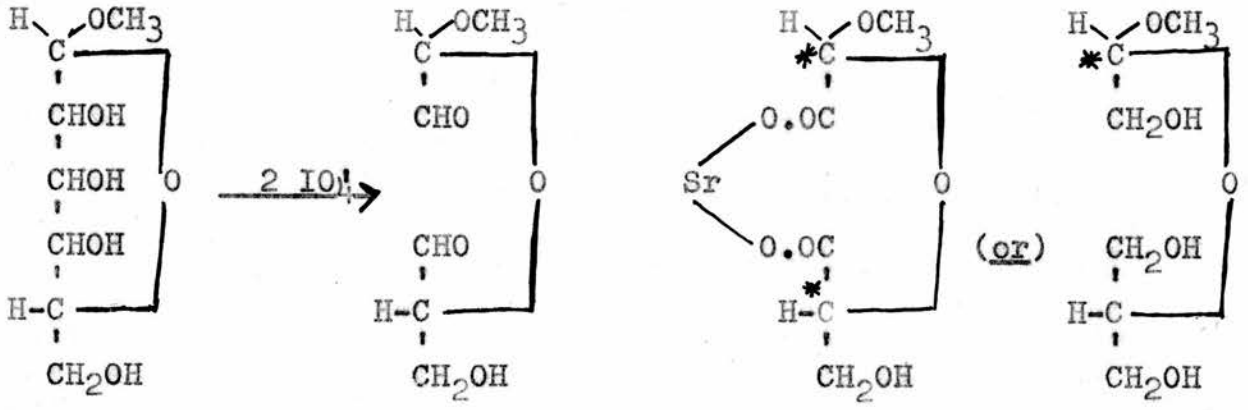
(11)

D-Galactosan

It has similarly been found that partly methylated sugars are not attacked by periodate in accordance with the simple theory of 1:2-glycol oxidation (14). To explain anomalous periodate uptake in these cases, it has been postulated that besides the simple oxidation of contiguous hydroxyl groups, periodate also attacks 'active' hydrogen atoms, converting them into hydroxyl groups which are then susceptible to further attack. Even this extension of the action of periodate fails to explain all the results from methylated sugars, and a number of explanations have been advanced (14).

Despite such anomalous examples, periodate oxidation has found extensive analytical and preparative use in carbohydrate chemistry. In the methyl hexopyranoside series, oxidation of the sixteen possible D-isomers can lead to only two products, one from methyl-α- and one from β-glycosides, thus offering a ready means of determining the anomeric configuration of any particular glycoside (15). The resultant aldehydic products (111) were originally converted to the dicarboxylic acid and isolated as the crystalline strontium salts (1V), which

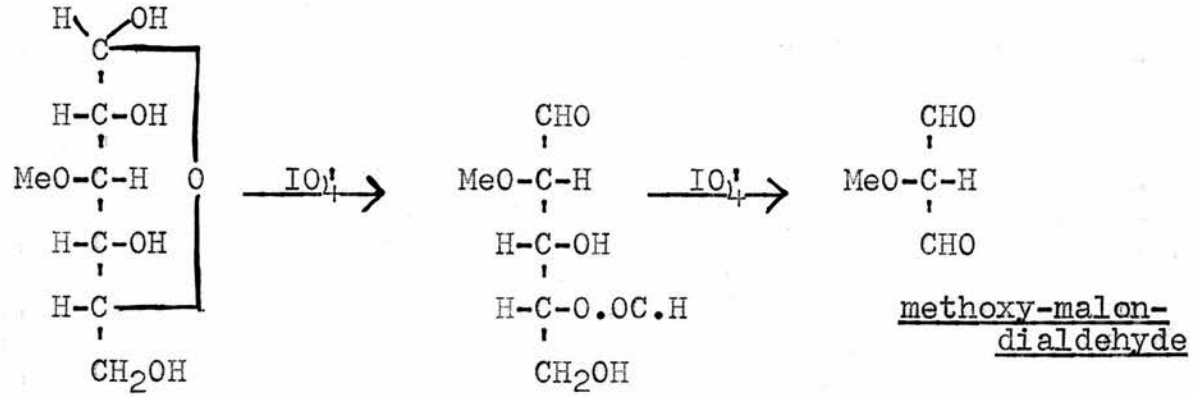
contanied two asymmetric C-atoms; a novel modification of this method consists in catalytic reduction of the end-product, thereby affording a compound with one asymmetric C-atom (V) :



Methyl-glyco-pyranoside (III) (IV) (V)

In structural studies on sugars and their derivatives, periodate oxidation has proved of value also in determining the ring structure of monosaccharides and the mode of linkage of oligosaccharides and polysaccharides (16). In carrying out such reactions the original method of Fleury (3) is still largely used to determine periodate uptake. Considerations of optimum pH, together with greater ease of analysis of end-products, has led to the use of sodium or potassium periodate in place of the original periodic acid. The procedure involves quantitative determination of periodate uptake and the amount of formic acid and formaldehyde released, together with, preferably, isolation and investigation of the end-product. In interpreting the results, care must be taken that oxidation is neither in excess of the theoretical uptake, nor incomplete due to unexpected side reactions. In this respect it is worth noting that formation of formyl esters can stop the action of periodate

thus leading to apparent incomplete oxidation and erroneous interpretation of analytical results (17).

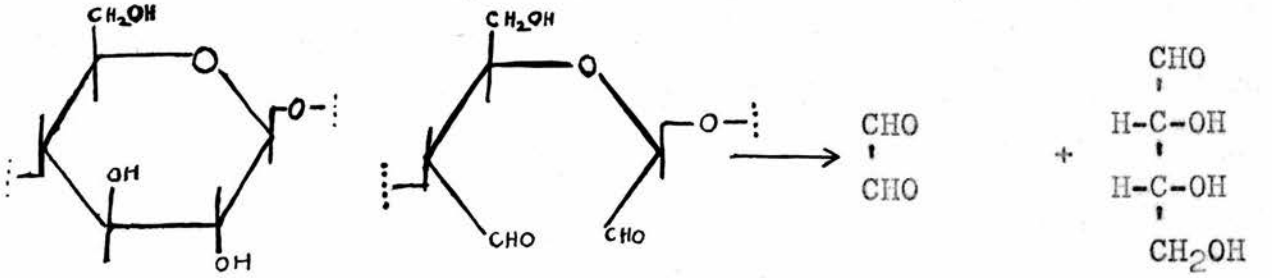


3-O-methylglucose

2-O-methyl-4-formyl arabinose

methoxy-malon-dialdehyde

The basic 1:4-glucopyranosyl repeating unit of cellulose (V1), having contiguous hydroxyl groups on C_2 and C_3, affords a structure in which each of the anhydro residues is vulnerable to periodate attack. Hydrolysis of oxidised cellulose (V111) should thus lead to the entire degradation of the molecule into glyoxal (V111) and erythrose (1X). It is not possible at present to state whether the small amount of glucose reported in the hydrolysate of oxo-cellulose is due to an anomalous linkage, incomplete oxidation, or the possible action of steric factors as in D-glucosan (18)



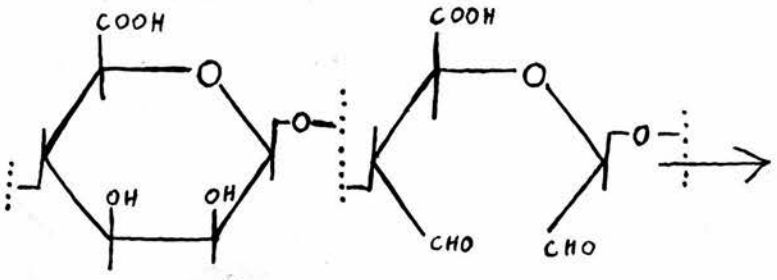
Cellulose (V1)

Oxo-cellulose (V11)

Glyoxal (V111)

Erythrose (1X)

Alginic acid (X), until recently considered to consist entirely of 1:4-linked mannuronic acid residues, gave an oxopolysaccharide (X1) which on hydrolysis was cleaved into glyoxal and erythruronic acid (X11) (19), (cf. Part 1, this thesis, page 12).



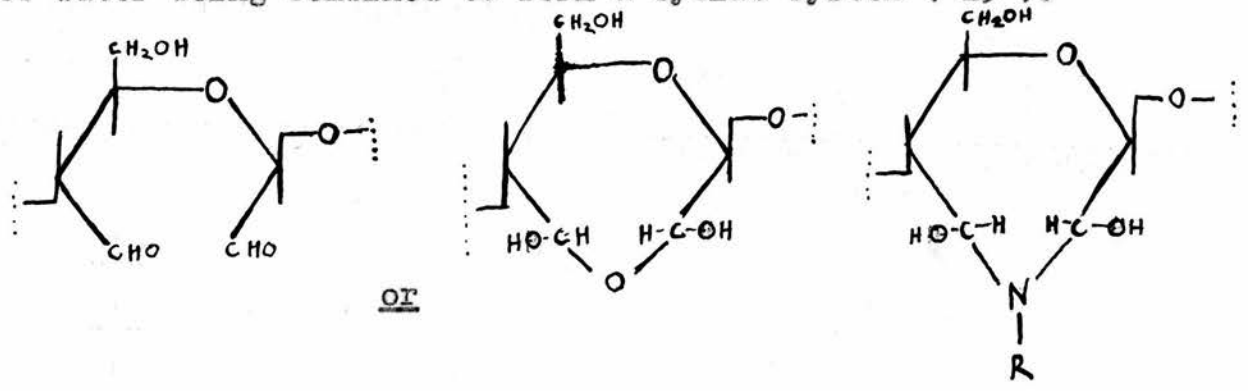
Alginic acid (X) — Oxo-product (X1) — Glyoxal — Erythruronic acid (X11)

In both these polysaccharides, the basic repeating unit consumed one mole of periodate, and no formic acid was produced. On the other hand, 1:6-linked hexopyranose residues are attacked between C_2, C_3 and C_4, with the consumption of two moles of periodate and the release of one mole of formic acid per repeating unit. Measurements on periodate uptake and formic acid release have been used to determine the relative proportions of 1:4- and 1:6-linkages in polysaccharides where both are present (20).

1:3-linked polysaccharides, having no glycol groups in the basic repeating unit, are open to periodate attack only at both ends of the polysaccharide chain (21). Measurement of periodate uptake and formic acid release in such cases are of value in determining chain-length, although caution must be exercised in postulating the mechanism of the reaction at the reducing end of the chain. In such molecules hydrolysis of

the oxopolysaccharide affords the basic monosaccharide unit in high yield, together with aldehydic fragments from the reducing and non-reducing ends of the chain.

Although oxocellulose (XIII) has been pictured as having two aldehydic groups per repeating unit, spectrophotometric examination of the oxidised polysaccharide gives no evidence for the presence of carbonyl groupings (22). In similar fashion analysis of the oxidation product of methyl D-glucopyranoside (III) has shown that the derived crystalline material has a molecule of condensed water, and gives no spectroscopic evidence for the presence of free aldehydic groups (23). Condensation of oxoraffinose in the cold with p-nitrophenylhydrazine affords a product with only half the quantity of nitrogenous base which might have been expected on the simple 'dialdehyde' theory (24). To explain such results, it has been postulated that the oxidation products are really hemi-aldehydic in nature, one molecule of water being combined to form a cyclic system (25).

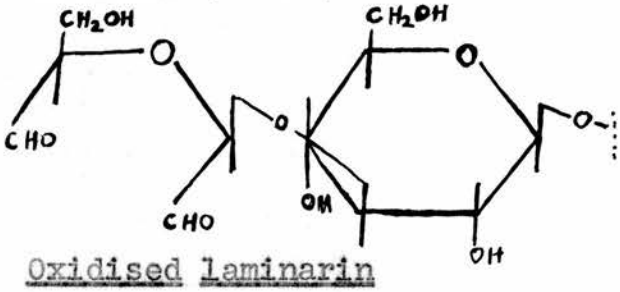


(XIII) (XIV) (XV)

In keeping with this hypothesis, it has been found that oxopolysaccharides, such as oxocellulose (XIV) will form amorphous condensation products (XV) with

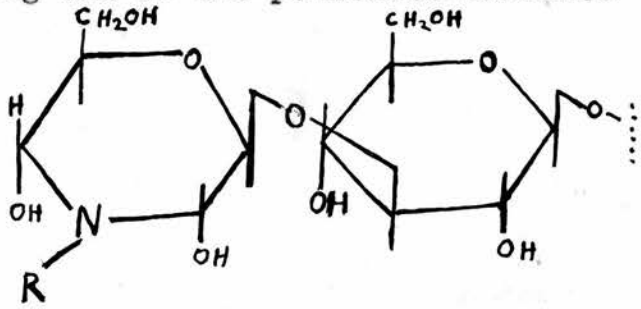
nitrogenous bases, in which only one amino grouping has reacted with each apparent dialdehyde in the oxidised material.

The amount of nitrogen in the condensation product of the 1:3-linked polysaccharide laminarin has been used to calculate the chain-length of the molecule (26). In this calculation the assumption was made that condensation took place only at the non-reducing end of the periodate-oxidised material (XVI) :-



Oxidised laminarin

(XVI)



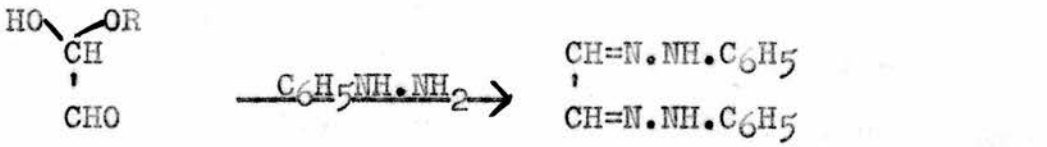
Condensation product

(XVII)

Among the nitrogenous bases used in such condensations are isoniazid, thiosemicarbazide, and p-aminobenzoic acid; these materials have been condensed in the cold with, for example, oxo-starch, oxo-alginic acid, and oxo-irisin (26).

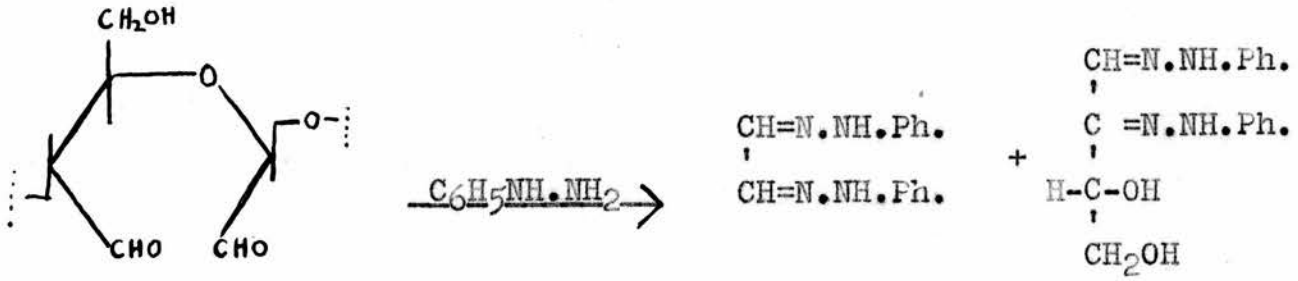
In the original investigations on oxocellulose, the polysaccharide after oxidation was hydrolysed with N acid for several hours, and the glyoxal produced identified as the bis-phenylhydrazone by addition of phenylhydrazine to the hydrolysate solution (27). Glyoxal was identified in similar fashion among the hydrolysis products of oxolaminarin (28). Soon afterwards it was discovered, by Barry and co-workers, that glyoxal bisphenylhydrazone could be isolated by merely heating a solution of the oxopolysaccharide with phenylhydrazine under weakly acid conditions (29) (XVIII).

This discovery bears comparison with the observation of Harries that glyoxal hemi-acetal affords glyoxal bis-phenylhydrazone on heating with phenylhydrazine; in somewhat similar fashion, 2-O-methylgalactose affords galactosazone (30).



Glyoxal hemi-acetal — Glyoxal bisphenylhydrazone

Degradation by phenylhydrazine has been applied, for example, to oxopolysaccharides from starch, inulin and cellulose (31). The procedure is to be carefully distinguished from the condensation reaction referred to earlier; the essential difference being that condensation products are formed by admixture of two cold solutions, while degradation is carried out by heating under mildly acid conditions. The difference is analogous to that in the preparation of simple phenylhydrazones and osazones.

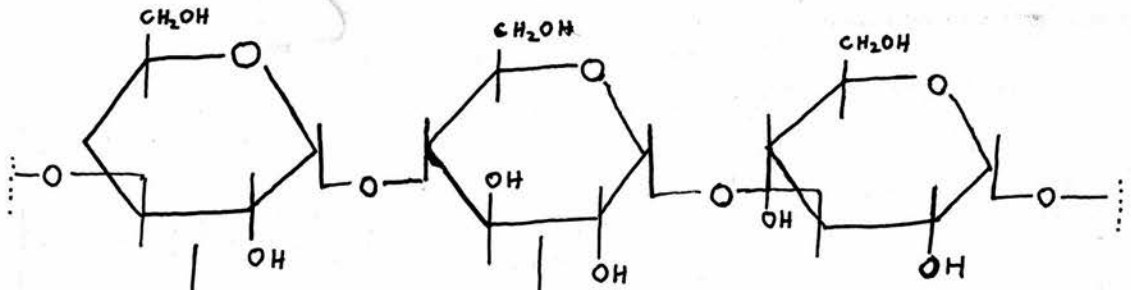


Oxocellulose — Glyoxal bis phenylhydrazone (XVIII) — Erythrosazone (XIX)

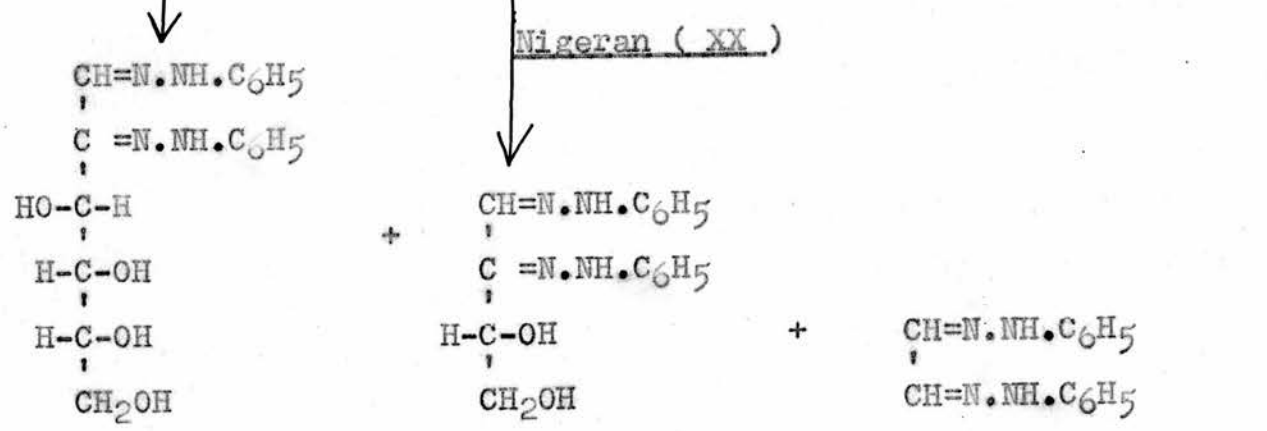
1:4-linked polysaccharides such as cellulose should, on degradation, afford glyoxal bisphenylhydrazone

(XVlll) and erythrosazone (XlX) in high yield. These products have indeed been isolated from degradation solutions, chromatography on alumina proving particularly advantageous in the isolation of closely-related compounds (31).

Oxidation and degradation of the polysaccharide nigeran (XX), which contains alternating 1:3- and 1:4-linked units, afforded glucosazone from those units which, because of the linkage on C_3, had been unattacked by periodate (32).



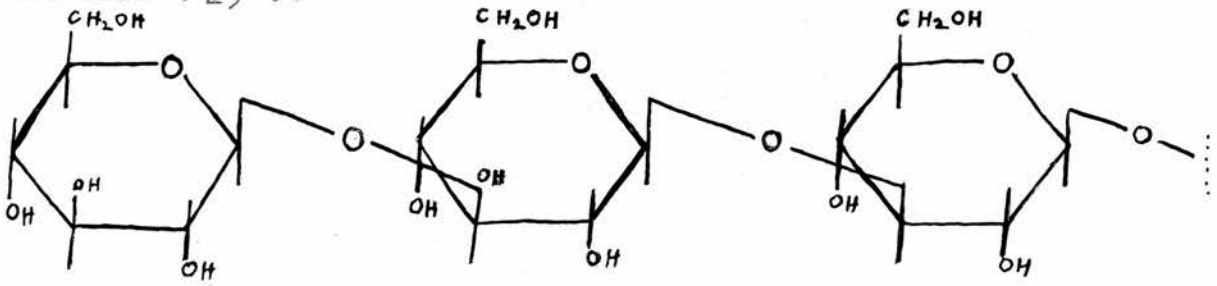
Nigeran (XX)



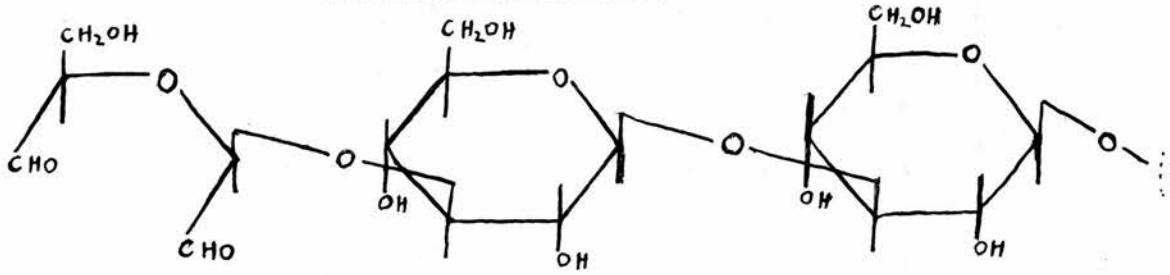
Glucosazone + Erythrosazone + Glyoxal-bis-phenyl-Hydrazone

In polysaccharides such as laminarin (XXl), where the basic repeating unit is a 1:3-linked hexopyranose unit, only units at the end of the chain are open to periodate attack and subsequent degradation. By heating the oxopolysaccharide (XXll) with phenylhydrazine, glycerosazone and glyoxal bisphenylhydrazone are removed from the non-reducing end of the polysaccharide; the molecule is thus left minus one

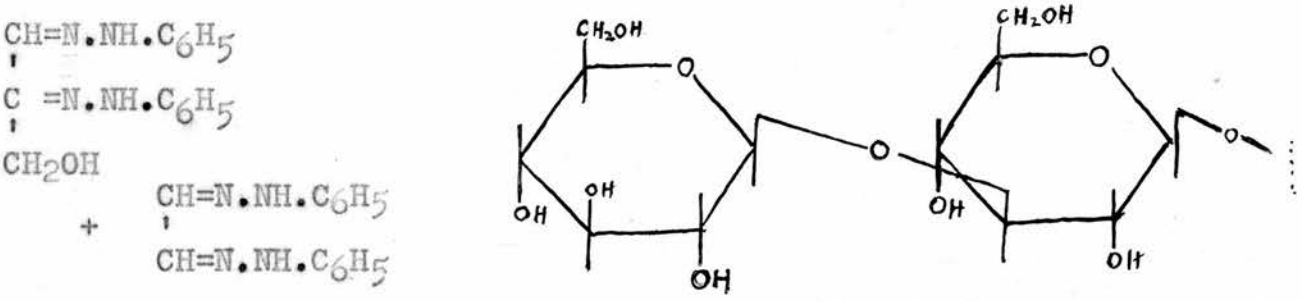
terminal unit at the non-reducing end, and with a modified grouping at the reducing end. Such a degraded polysaccharide is now open to periodate attack and degradation as before. Ten consecutive degradations have been carried out on yeast glucan (a supposed 1:3-linked glucose polymer), the procedure being essentially similar in each case (29).



Laminarin (XXI)



Oxopolysaccharide (XXII)



Glycerosazone + Glyoxal bis-phenylhydrazone + Degraded laminarin (XXIII)

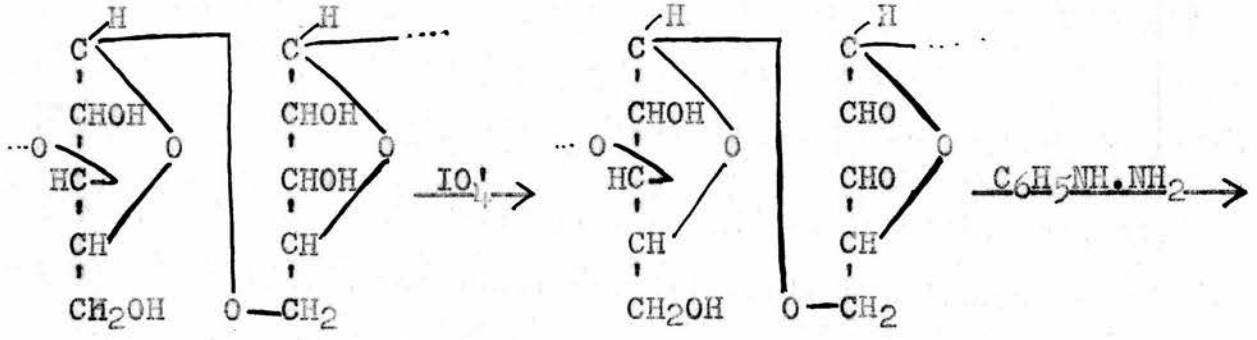
In more complicated molecules,(33) where several sugars and/or different types of linkages are present, the Barry degradation procedure has proved of value in distinguishing between several possible structures arrived at from other experimental data. Arabic acid (a degraded form of gum arabic) has been

investigated by this method with a view to elucidation of structure (34). The formula initially proposed for this molecule envisaged a backbone of galactose units containing alternate 1:3- and 1:6-links (35). Such a molecule would, after removal of side-chains, be broken up into single sugar units as in the case of nigeran. Instead it has been found that after three degradations, arabic acid affords a molecule containing only galactose which cannot be further degraded to any appreciable extent. The existence of such a residue is incompatible with the earlier proposed structure.

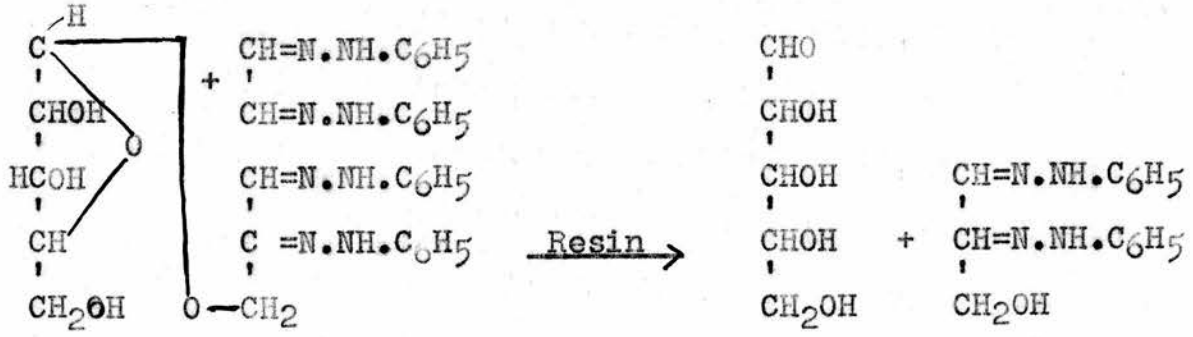
In like manner the polysaccharide extract fron the red weed, Dilsea edulis, which contains minor quantities of D-xylose and D-glucuronic acid (ca. 10%) along with D-galactose (ca. 70%), has been subjected to several consecutive degradations(36). The xylose and uronyl units were removed during the first degradation, indicating that these residues are attached to the main bulk of the molecule as side-chains near the periphery. Three further degradations afforded a polysaccharide which still contained galactose, indicating that at least some of these units are linked in the 1:3 position.

All stages of the polysaccharides after degradation contain a certain amount of inseparable nitrogen (1 - 5%). While it has been postulated that at least some of the nitrogen may be due to adsorbed phenylhydrazine (52), it appears more likely that it is chemically bound to aldehydic groups in the polysaccharide. Thus for example, where 1:6-linked side-chains are joined to a central resistant backbone,

oxidation and degradation might reasonably be expected to leave a bis-phenylhydrazone fragment attached to the degraded material. The existence of such fragments has in fact been proved (37), and is in keeping with the general properties of sugar phenylhydrazones; such compounds exhibit a strong tendency to add on one more phenylhydrazine residue, thereby affording osazones, but the reaction does not proceed beyond this stage. The hydrolytic action of base-exchange resin on the glycosidic linkage of disaccharide osazones should, if applicable to polysaccharides, prove of advantage in obtaining nitrogen-free degraded materials, and in elucidating complex structures (38)



Polysaccharide containing 1:5-linked arabofuranose units



Arabinosyl-glycerosazone Arabinose + Glycerosazone

Summarising then, the Barry degradation procedure is likely to afford structural information not readily determinable by other methods. It should be possible, by methylation at each oxidised and degraded stage, to arrive at an unambiguous picture of the types of linkage in a complex polysaccharide. Such a polysaccharide is Cladophoran (39), and it was in an attempt to elucidate more structural details for this polymer that the experiments described below were initiated.

EXPERIMENTAL

EXPERIMENTAL

(a): Barry Degradation of Laminarin

Nomenclature : The state of the polysaccharide under discussion, i.e. oxidised or degraded, is denoted by the suffixes O and D respectively, the appended numerals indicating the number of such reactions to which the polysaccharide has been subjected. Thus, for example, cladophoran which has been oxidised once and then degraded is referred to as cladophoran O_1D_1; similarly laminarin which has been oxidised three times (and therefore of necessity degraded twice) is referred to as laminarin D_2O_3.

Since laminarin consists mainly of 1:3-glucopyranosyl units , it can be regarded as a model substance on which to carry out an evaluation of the technique of degradation, and investigations on this polysaccharide are therefore described first. In calculating percentage yields of oxopolysaccharide, allowance is made for material consumed during measurements on the course of the reaction.

First Oxidation of Laminarin

The polysaccharide (4.16 g.) was dissolved in warm water (50 ml.) and, after cooling, an equal volume of sodium periodate solution (0.190 M) was added. The mixture was shaken over several days, samples being withdrawn for analysis at the intervals described below. To measure the acidity produced, samples (1 ml.) were treated with excess of ethylene glycol and, afterstanding for ten minutes, were titrated against

sodium hydroxide solution (0.01 M), using methyl red indicator (41). To measure periodate uptake, samples of the reaction mixture (1 ml.) were added to arsenite solution (5 ml., 0.05 N), and the mixture treated with sodium bicarbonate solution (1 ml., 2%), together with a crystal of potassium iodide. After ten minutes, the residual ~~iodine~~ arsenite was titrated against a solution of iodine (0.0114 N), using starch indicator. (40).

5 ml. of arsenite soln. = 21.9 ml. of iodine soln.

5 " " " " +
0.5 ml. of periodate soln = 5.1 " " " " .

Time (hr.)	1.25	3.5	8	25	32	48	75	96
0.01 N NaOH (ml.)	1.56	1.90	2.04	2.48	2.60	2.72	2.84	3.16
0.0114 N I_2 (ml.)	10.9	11.6	12.6	13.4	13.7	14.5	15.3	15.5

After 96 hours :-

Amount of periodate consumed =

1 mole per 710 g. of polysaccharide.

Amount of acid produced =

1 mole per 1230 g. of polysaccharide.

Oxidation was then stopped by passing sulphur dioxide through the cooled reaction mixture. Following dialysis, the residual polysaccharide was isolated by freeze-drying (3.04 g., 84%).

First Degradation of Laminarin

The oxopolysaccharide (2.86 g.) was dissolved in water (70 ml.), and phenylhydrazine (2 ml.) and

acetic acid was added (5 ml.). The mixture was heated at 100° for two hours, a golden-brown precipitate being obtained on cooling Following exhaustive extraction with ether, the polysaccharide remaining in the water phase was isolated on freeze-drying as a light-yellow powder (2.28 g.; 79%) (Found: N, 0.94%). A further extraction with ether did not lower the nitrogen content. Hydrolysis of the polysaccharide and estimation of the reducing power by the Schaffer-Somogyi method (42) gave a value of 86.4% conversion to glucose; the corresponding value for the original laminarin was 93%.

Second Oxidation of Laminarin

Degraded laminarin (1.34 g.) was treated with periodate solution (40 ml., 0.100 M), 1-ml. samples being analysed for uptake of oxidant and production of acid. In this instance the amount of periodate present was estimated by direct titration against a solution of sodium thiosulphate (0.05 N); measurements of acidity were carried out as hitherto described.

1 ml. of periodate soln. = 16.0 ml. of thiosulphate soln.

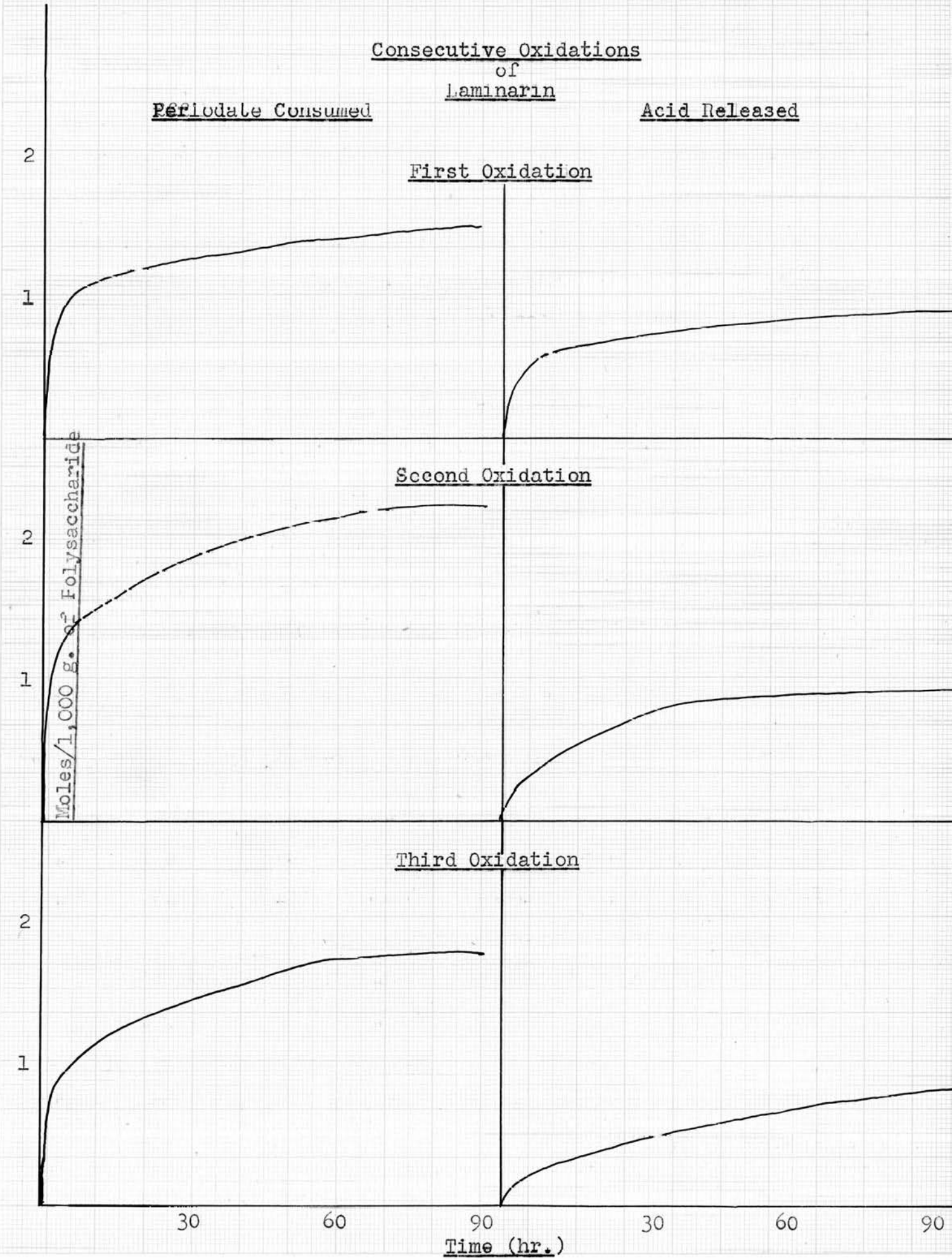
1 ml. of periodate soln. + glycol = 12.0 ml. of thiosulphate soln.

Time (hrs):	1	4	22	50	72	96
0.01 N NaOH: (ml.)	0.50	1.09	1.74	2.33	2.67	2.72
0.05 N $Na_2S_2O_3$: (ml.)	15.4	14.2	13.74	13.0	12.8	12.8

After 96 hours :-

Uptake of Oxidant = 1 mole per 420 g. of polysaccharide.

Production of Acid = 1 mole per 1230 g. of polysaccharide.


Consecutive Oxidations
of
Laminarin
Periodate Consumed
Acid Released
First Oxidation
Second Oxidation
Third Oxidation
Moles/1,000 g. of Polysaccharide
2
1
2
1
2
1
30
60
90
30
60
90
Time (hr.)

Oxidation was then stopped and the solution dialysed as before. Addition of alcohol afforded oxopolysaccharide (0.74 g.) as a light brown powder (Found : N, 0.51%). The oxidised material (528 mg.) was treated with acetic acid (1 ml.) and phenylhydrazine (0.4 ml.) as before; the degraded polysaccharide was isolated in 87% yield (478 mg.; Found: N, 1.01%).

In a second experiment on oxidant uptake, using the arsenite method of estimation, a value of one mole per 480 g. of polysaccharide was obtained.

Third Oxidation of Laminarin

Twice-degraded laminarin, O_2D_2 (397 mg.) was treated with periodate solution (0.094 M, 20 ml.); the course of reaction was followed in the usual manner.

5 ml. of arsenite soln. (0.05 N) = 28.2 ml. of iodine (0.0089N)

1 ml. of periodate soln. (0.094 M) = 7.1 " " " " .

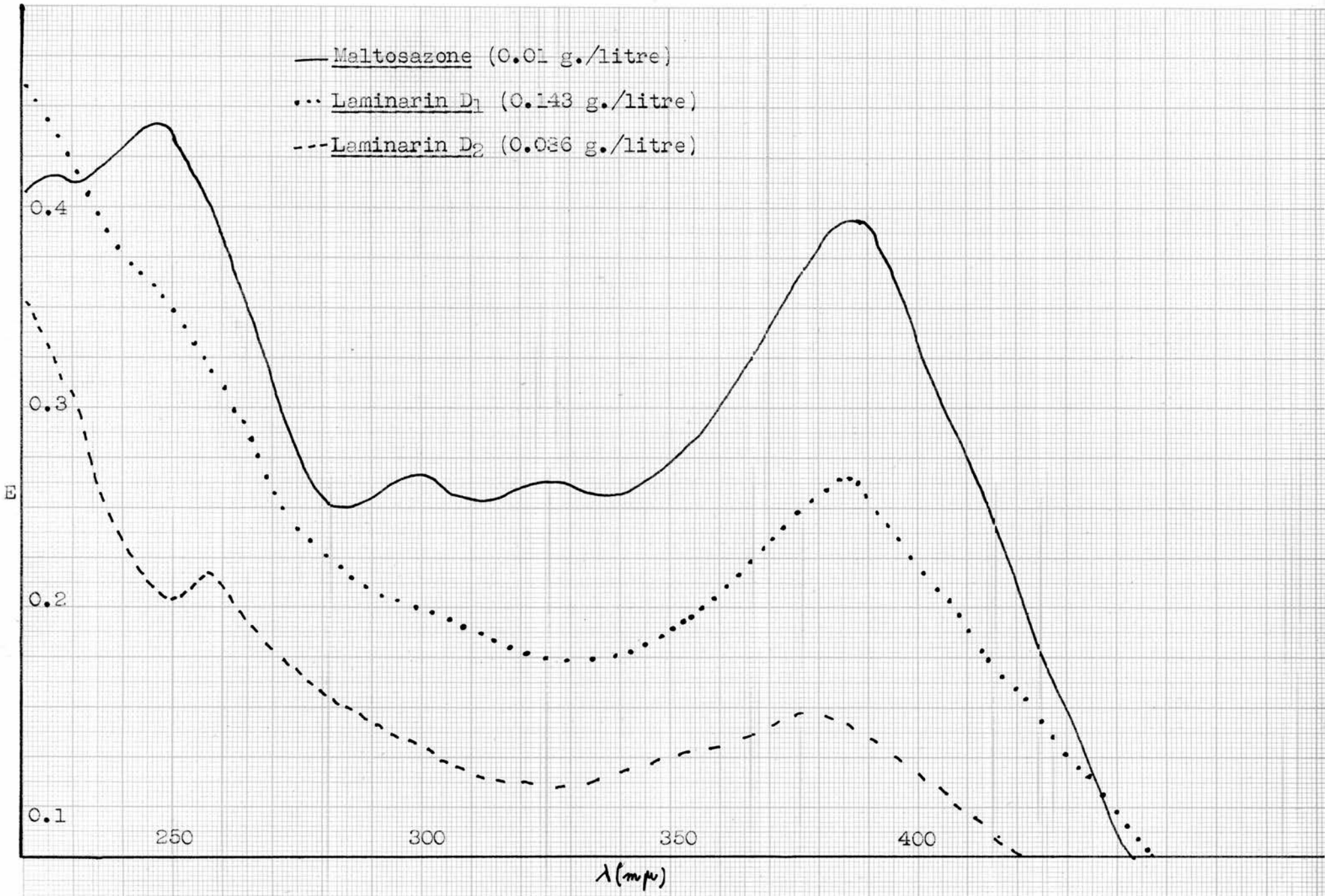
Time (hrs.):	1	6.5	22.5	47.5	74	95
0.01 N NaOH (ml.):	0.20	0.26	0.38	0.94	1.32	1.48
0.0089 I_2 (ml.):	11.20	12.36	12.68	13.84	14.26	14.42

After 96 hours :-

Oxidant consumed = 1 mole per 610 g. of polysaccharide

Acid produced = 1 mole " 1380 g. " " .

Oxidation was then stopped and the oxopolysaccharide (118 mg., 76%) isolated as a light brown powder (Found: N, 0.48%).


—Maltosazone (0.01 g./litre)
··· Laminarin D1 (0.143 g./litre)
---Laminarin D2 (0.036 g./litre)
0.4
0.3
0.2
0.1
E
250
300
350
400
λ(mμ)

U.V. Absorption of the Degraded Polysaccharides (43)

A solution of laminarin O_1D_1 was made up in water at a concentration of 0.143 g./litre, and its absorption spectrum in the range 220 - 450 mμ. measured in a Beckmann U.V. Spectrophotometer. A solution of maltosazone (c., 0.01 g./litre) was used as a reference substance, the following results being obtained (cf. page 113):-

A/ Laminarin O_1D_1 (c., 0.143 g./litre) exhibited an absorption band of intensity 0.267 at $\lambda_{max.}$ 383 -385 mμ.

B/ Laminarin O_2D_2, similarly examined at c., 0.086 g./litre, exhibited two peaks:-

(1) - of intensity 0.139 at $\lambda_{max.}$ 370 - 371 mμ.

(2) - of intensity 0.218 at $_{max.}$ 257 - 259 mμ.

C/ Laminarin D_1O_2 and D_2O_3 exhibited non-characteristic absorption curves, with no distinguishing peaks.

Reprecipitation of Laminarin

Each of the polysaccharides isolated at the stages described above exhibited the phenomenon of delayed reprecipitation from solution, characteristic of the original polysaccharide. They were readily soluble in warm water; precipitation did not take place immediately on cooling, but gradually over a period of up to ten hours.

Barry Degradation of Cladophoran

The polysaccharide extract from Cladophora rupestris was prepared as described by Percival and Fisher. The isolated material was obtained as a fine white powder (Found: Ash, 15.7; sulphate, 18.3, N, 1.7%). Hydrolysis of a sample (5 hr., N H_2SO_4) confirmed the presence of the sugars galactose, glucose, arabinose, xylose and rhamnose; as determined by the above authors, the approximate molar proportions of these sugars were as follows :-

Arabinose	galactose	xylose	rhamnose	glucose
4.0	3.0	1.08	0.5	0.23 .

First Oxidation of Cladophoran

The polysaccharide (26.3 g.) was dissolved in water (500 ml.) and an equal volume of sodium periodate (0.196 M) was added. Measurements on oxidant uptake and production of acid were carried out as described in the case of laminarin. A small correction (= 0.02 ml. of alkali) was made to allow for the acidity of the polysaccharide.

5 ml. of arsenite soln. (0.05 N) = 26.9 ml. of iodine soln. (0.0093 N)
5 " " " " " + = 5.6 " " " " " .
0.5 ml. of periodate soln.

Time (hr.)	0.75	3	6	22.5	30	50.5	57 .
I_2 (0.0093 N, ml.)	13.5	15.9	18.5	20.8	21.2	21.5	21.6
NaOH (0.01 N, ml.)	1.05	2.79	3.72	4.02	4.25	4.70	4.76

After 57 hours :-

Periodate uptake = 1 mole per 360 g. of polysaccharide

Acid produced = 1 " " 550 g. " " .

Oxidation was then stopped and, following dialysis, the solution of oxopolysaccharide was made up to 1250 ml. An aliquot (50 ml.) of this solution afforded 0.91 g. of material on freeze-drying (87% yield) (Found: Ash, 15.0; sulphate, 19.2; N, 1.52%) ($[\alpha]_D$ + 35°(c. 1.8)). Hydrolysis of a sample (6 hr., N H_2SO_4), and chromatographic estimation of the hydrolysate by the colorimetric method (44), revealed that the molar proportions of the constituent sugars were as follows :-

Arabinose	galactose	rhamnose	glucose
4.0	0.86	0.67	0.35 .

Degradation of the Oxopolysaccharide

The oxidised material (23 g. in 1.2 litres of water) was heated with phenylhydrazine (20 g.) and acetic acid (50 ml.) at 100° for two hours; a heavy brown precipitate gradually settled out during the course of the reactin. After exhaustive extraction with ether, followed by dialysis, freeze-drying afforded the degraded polysaccharide as a brown powder (13.7 g., 67%). It had ash 15.6; sulphate 16.6; N 3.45%. A solution of the polysaccharide in water was too yellow to permit of rotational measurements; spectroscopic examination of the material, as in the case of laminarin, revealed one absorption band of intensity 0.312 at $\lambda_{max.}$ 360 - 363 (c. 0.142 g./litre).

The hydrolysate of a small

sample was chromatographically indistinguishable from that of the oxopolysaccharide.

Examination of the Ether Extract

The ethereal solution obtained by exhaustive extraction of the degraded polysaccharide was evaporated to small volume and examined by means of circular chromatography (45) (solvent = toluene : water : ethanol :: 270 : 30 : 1 (v/v)). Two bands corresponding to glyoxal bisphenylhydrazone and N-acetyl phenylhydrazine were observed. The solution was therefore evaporated to dryness and treated with 50 ml. of acetic acid. The insoluble portion was centrifuged off and dissolved in a minimum of ether. Addition of light petroleum afforded pale yellow crystals of glyoxal-bis-phenylhydrazone, m.p. 167° (0.9 g.).

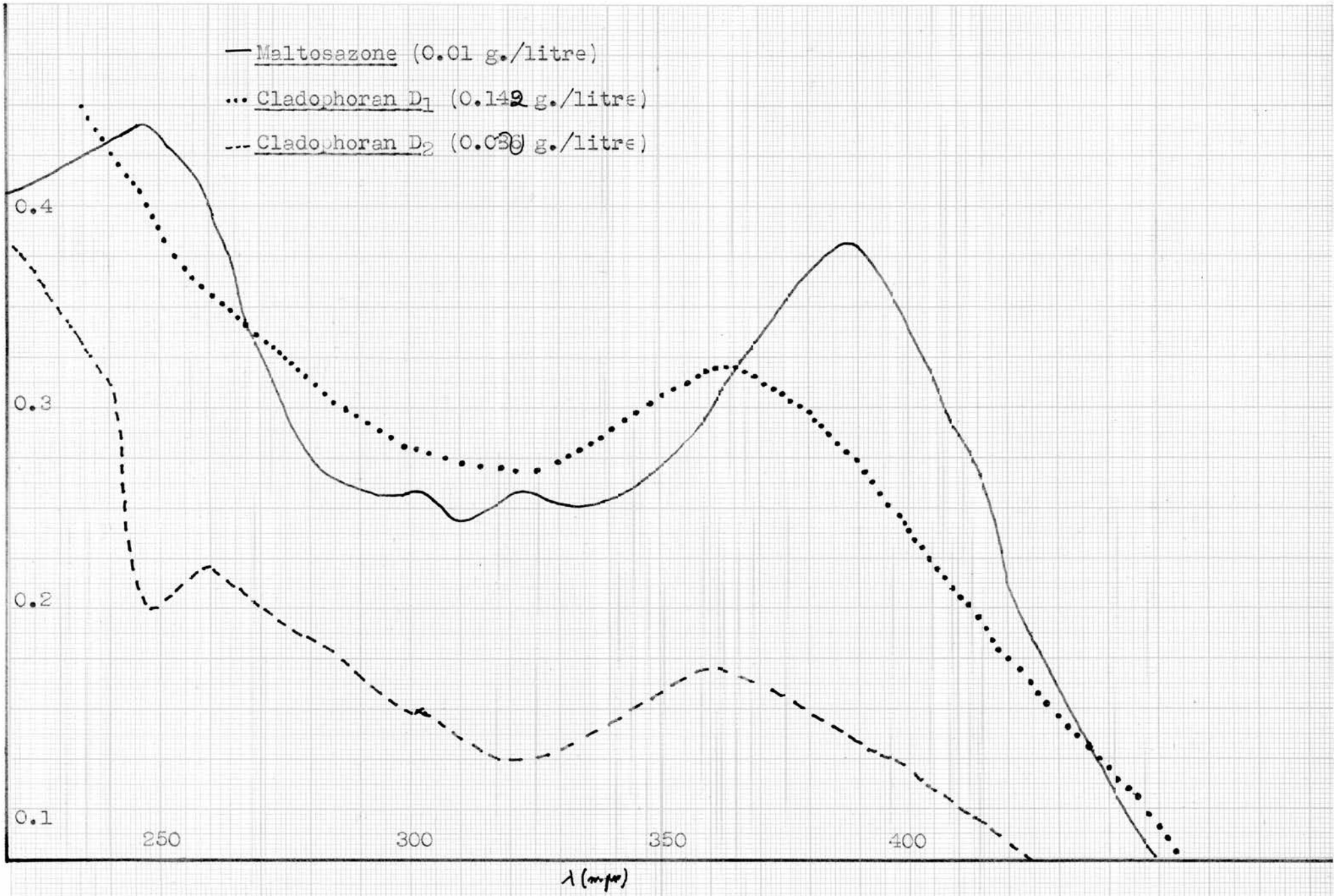
Evaporation of the supernatant acetic acid solution, followed by recrystallisation of the residue from ether, yielded a large quantity of N-acetyl-phenylhydrazine, m.p. 124°.

Second Oxidation of Cladophoran

The degraded polysaccharide O_1D_1 (12.83 g.) was dissolved in water (300 ml.) and an equal volume of sodium periodate solution (0.108 M) was added. Samples (2 ml.) were removed at intervals and the course of reaction followed as hitherto described.

5 ml. of arsenite soln. (0.05 N) = 27.2 ml. of iodine soln. (0.0092M)

5 " " " " " +
1 ml. of periodate soln. (0.108M) = 3.8 " " " " " .


— Maltosazone (0.01 g./litre)
··· Cladophoran D1 (0.142 g./litre)
--- Cladophoran D2 (0.036 g./litre)
0.4
0.3
0.2
0.1
E
250
300
350
400
λ (mμ)

Time (hr.)	1	4.5	21	28	45	52	71
I_2 (0.0092 N, ml.)	12.8	15.1	20.8	21.6	21.9	21.9	22.0
NaOH (0.01N, ml.)	1.00	1.31	1.68	1.89	2.31	2.36	2.48

After 71 hours :-

Periodate uptake = 1 mole per 510 g. of polysaccharide

Acid produced = 1 " " 1760 g. " " .

Oxidation was then stopped and an aliquot portion of the oxopolysaccharide, cladophoran D_1O_2, was isolated. It was thus calculated that the total yield was 9.9 g. (80%). The material had Ash, 12.3; sulphate, 17.5; N, 2.7%. Estimation of the sugar content as in the case of cladophoran O_1 gave the following molar proportions :-

Arabinose : galactose : rhamnose
4.0 : 0.89 : 0.65 .

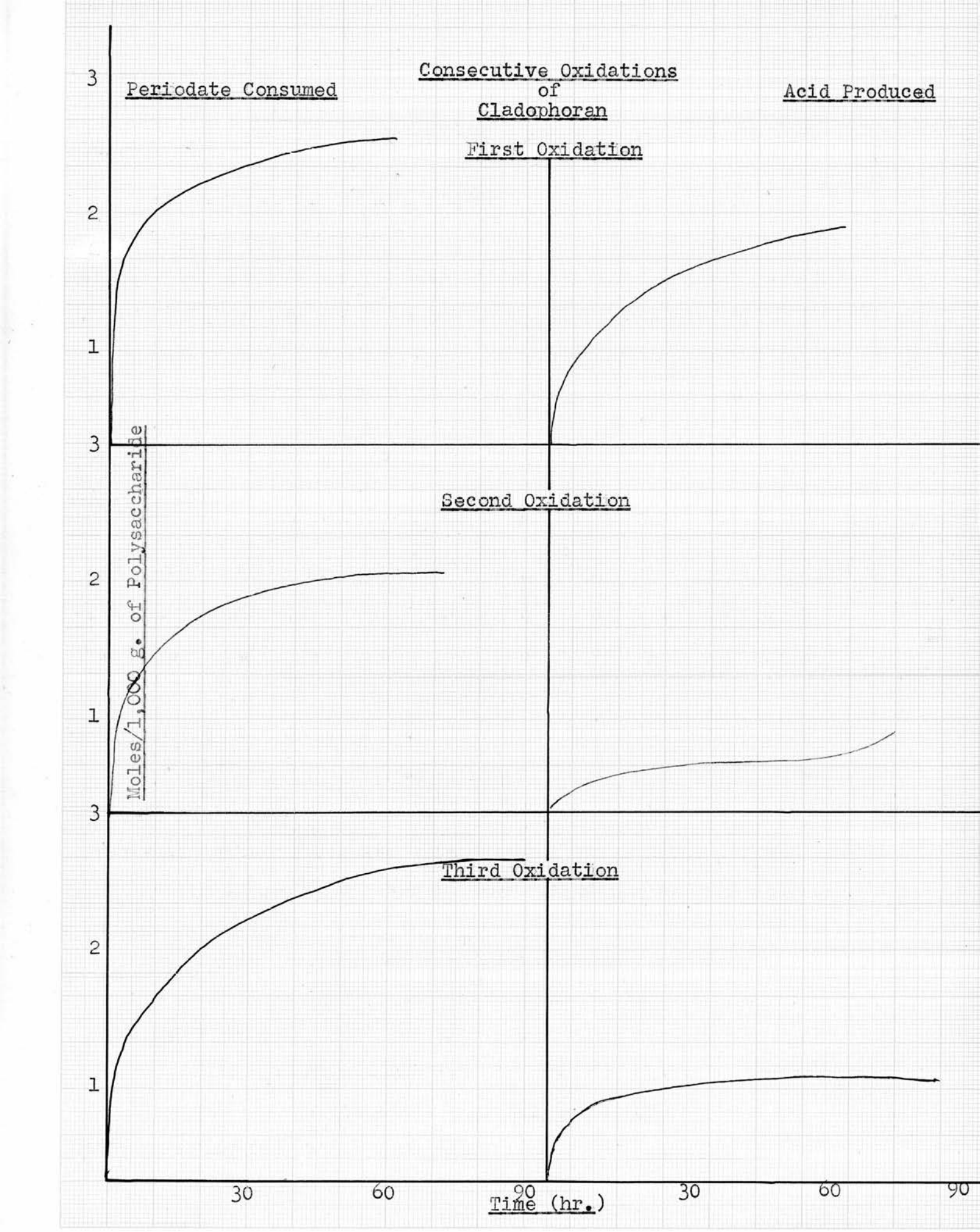
Second Degradation of the Polysaccharide

Cladophoran D_1O_2 (9.5 g. in 1250 ml. of water) was heated with phenylhydrazine (10 g.) and acetic acid (25 ml.) at 100° for two hours. Following ether extraction and dialysis, the degraded polysaccharide was isolated as before (6.3 g., 66%) (Found: Ash, 12.8; sulphate 16.9; N, 4.2%) Spectroscopic examination (c. 0.0676 g./litre) revealed the presence of two absorption peaks:-

(a) - of intensity 0.168 at $\lambda_{max.}$ 357 - 359 mμ.

(b) - of intensity 0.218 at $\lambda_{max.}$ 258 - 260 mμ.

A sample hydrolysate was chromatographically indistinguishable from that of the oxidised material, cladophoran D_1O_2.


Consecutive Oxidations
of
Cladophoran
Periodate Consumed
Acid Produced
First Oxidation
Second Oxidation
Third Oxidation
Moles/1,000 g. of Polysaccharide
3
2
1
3
2
1
3
2
1
30
60
90
30
60
90
Time (hr.)

Third Oxidation (small-scale)

A sample (0.529 g.)of the twice-degraded polysaccharide, O_2D_2, was treated with periodate solution (30 ml., 0.095 M), and the course of reaction followed by withdrawal of samples (1 ml.) at intervals. (Accurate estimation of the acid end-point was made difficult owing to the yellow colour of the solution).

5 ml. of arsenite soln. (0.05 N) = 21.9 ml. cf iodine soln. (0.0114 N)
5 " " " " " +
1 ml. of periodate soln. (0.095 M) = 5.1 ml. " " " " .

Time (hr.)	1.25	3.5	8	25	32	48	75	96
I_2(0.0114 N, ml.)	7.4	9.3	10.2	11.4	11.9	12.8	12.8	12.9
NaOH (0.01 N, ml.)	1.2	1.4	1.6	1.7	1.8	1.9	1.9	1.9

After 96 hours :-

Periodate uptake = 1 mole per 390 g. of polysaccharide
Acid produced = 1 " " 930 g. " " .

The oxopolysaccharide (182 mg., 73%) was isolated in the usual manner (Found: N, 2.38%; sulphate 15.9%). Estimation of the sugar content of a sample hydrolysate gave :-

Arabinose : galactose : rhamnose
1.0 : 1.04 : 0.65 .

Degradation of this material afforded 132 mg. of polysaccharide (74%).

Fourth Oxidation of Cladophoran

The thrice-degraded polysaccharide,

O_3D_3, (132 mg.) was treated with periodate solution (20 ml, 0.100 M), and the uptake of oxidant measured after 96 hours :-

Uptake of oxidant = 1 mole per 290 g. of polysaccharide.

Oxidation was then stopped by passing sulphur dioxide through the cooled solution. On attempting to remove inorganic material by dialysis, it was found that the oxopolysaccharide, D_3O_4, was passing through the dialysis membrane. The solution was therefore made N with respect to sulphuric acid and hydrolysed at 100° for six hours. Following deionisation with IR-4B-OH and IR-120-H resins, chromatographic examination of the hydrolysate revealed the presence of arabinose, galactose and rhamnose.

Third Oxidation (large-scale)

Twice-degraded cladophoran, O_2D_2, (4.28 g.) was treated with periodate solution (200 ml., 0.094 M) during 96 hours. Oxidation was then stopped and, following dialysis, the oxopolysaccharide was isolated by freeze-drying(3.81 g., 89%) (Found: Ash, 13.4; sulphate, 15.2; N, 2.4%). Examination of the absorption spectrum of this material, together with that of cladophoran D_1O_2, gave non-characteristic curves in each case, with no distinguishing absorption maxima.

Methylation of Thrice-oxidised Cladophoran

Thallium hydroxide (6 g.) was added to a solution of cladophoran D_2O_3 in water (3.6 g., 30 ml.), and the mixture was freeze-dried. After refluxing overnight with

methyl iodide (25 ml.) and following evaporation to dryness, the residue was exhaustively extracted with methanol (3 x 25 ml.), hot 50% methanol (3 x 25 ml.), and with hot water (3 x 25 ml.). The combined extracts were treated with thallium hydroxide and methyl iodide as before. After four methylations in all, the final residue was exhaustively extracted with chloroform, yielding a brown polysaccharide material (1.88 g.) (Found: OMe 26.7%). Two treatments with silver oxide and methyl iodide did not raise the methoxyl value beyond 26.9%.

Hydrolysis of the Methylated Polysaccharide

Thrice-oxidised methylated cladophoran (1.8 g.) was hydrolysed for seven hours with N methanoli hydrogen chloride containing water (10%). Following neutralisation with silver carbonate, the mixture was evaporated to dryness. The residue was exhaustively extracted with water, this procedure being efficacious in leaving brown nitrogenous material adsorbed on the precipitated silver salts. Evaporation of the aqueous extract to dryness, followed by chloroform extraction of the residue and evaporation, gave an amorphous methylated hydrolysate (0.96 g.). This mixture of methylated sugars was applied to a cellulose column (55 x 2.3 cm.) and eluted with a water-saturated mixture of light petroleum ; n-butanol (8 ; 2). After elution of the mono-O-methylpentose fraction (cf. below), the proportions of the eluant mixture were changed to 7:3. R_G values are recorded for solvent (6); each fraction was purified by evaporation to dryness and aqueous extraction of the residue, the procedure being repeated several times.

Fraction 1 : 213 mg. ; R_G 1.05

Evaporation to dryness afforded a yellow syrup; the material evidently contained nitrogenous substances, possibly together with aldehydic fragments from the original oxopolysaccharide, cladophoran D_2O_3. In view of the fact that its R_G value did not correspond with that of any known sugar, the material was not further examined.

Fraction 2 : 40 mg. ; R_G 0.83

$[\alpha]_D$ - 17° (c. 0.3)

(Found: OMe, 33.4%; Calc. for di-O-methyldeoxyhexose, 32.3%). The clear syrup gave only rhamnose on demethylation. Nucleation with an authentic specimen afforded 2:4-di-O-methylrhamnose as thin needles; the crystals had m.p. 87°, undepressed by admixture with a specimen of m.p.88-9°.

Fraction 3 : 162 mg. ; R_G 0.75

$[\alpha]_D$ + 28° (c. 2.0)

(Found: OMe, 34.9; Calc. for di-O-methylpentose; OMe, 34.8%) Arabinose was the only sugar given on demethylation; the syrup was chromatographically identical with the 2:4-, as distinct from the 2:3- and the 2:5-isomers. Formation of the anilide derivative gave crystals of m.p. 139-40°; two anilide derivatives are quoted, with m.p. 145° and 126° respectively (46): for 2:4-di-O-methylarabinose.

Fraction 4 : 143 mg. ; R_G 0.64

$[\alpha]_D$ + 88° (c. 0.92)

(Found: OMe, 41.7; Calc. for tri-

O-methylhexose, 41.9%). The material gave galactose on demethylation; slow evaporation from methanol yielded crystals of m.p. 116°. Although the m.p. of 2:4:6-tri-O-methylgalactose is quoted as 105°, only the 2:4:6-isomer among the trimethylgalactoses has rotation($[\alpha]_D$ + 93°) approaching that of the sugar under discussion, (47).

Fraction 5 : 37 mg. ; R_G 0.43

$[\alpha]_D$ + 74° (c. 1.8)

(Found: OMe, 18.0; Calc. for mono-O-methylpentose, 18.8%). The syrup was chromatographically and ionophoretically identical with 2-O-methylarabinose; although a slight yellow coloration accompanying the pink spot (aniline oxalate spray) suggested that perhaps a second substance was present, in no instance was any indication of separation achieved. The identity of the sugar was also indicated as the 2-isomer by spraying with a reagent specific for such sugars (48); as with sugars substituted on C_2 in general, the 2-O-methylarabinose did not develop a characteristic red coloration.

Identity was conclusively proved by formation of the phenylhydrazone derivative, with m.p. 115°.

Fraction 6 : 89 mg. ; R_G 0.30

Purification with charcoal, followed by slow evaporation from methanol, afforded crystalline L-rhamnose hydrate, with m.p. and mixed m.p. 68°

Fraction 7 : 23 mg. ; R_G 0.23

$[\alpha]_D$ + 74° (c. 1.1)

(Found: OMe, 15.1; Calc. for mono-O-methylhexose, 16.0%). The syrup gave only galactose on demethylation, and was chromatographically identical with an authentic specimen of 6-O-methylgalactose. Identity was proved by formation of the sparingly-soluble phenylhydrazone, with m.p. 135° (49).

Fraction 8 : 46 mg. ; R_G 0.10

Purification with charcoal, followed by slow evaporation and nucleation, afforded crystalline D-galactose; it had m.p. and mixed m.p. 162-4°.

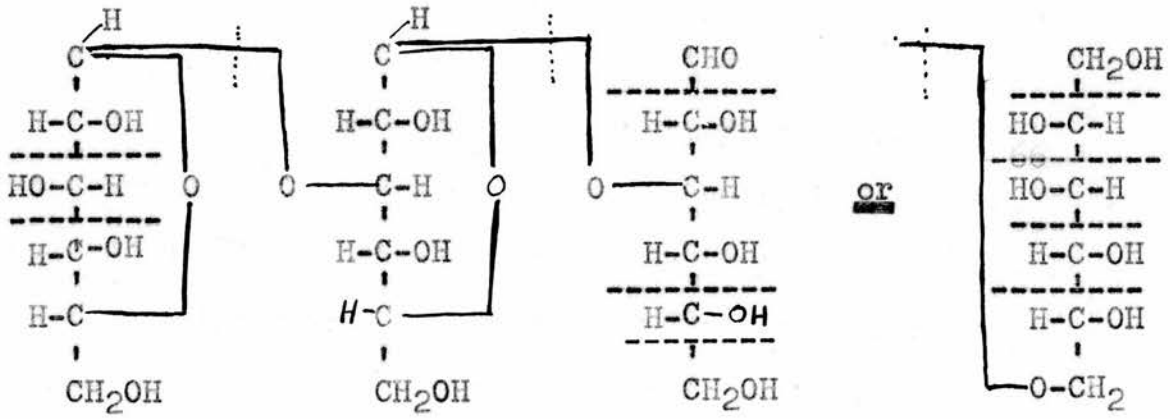
DISCUSSION

PART II (a) - DISCUSSION

Periodate Oxidation of Laminarin

The simple theory of the laminarin molecule requires a periodate consumption of two moles at the non-reducing end of the polysaccharide, together with three moles at the reducing end (cf. below). Although oxidation at the reducing end is often complicated by formation of formyl esters, in the experiments under discussion reaction times were sufficiently long to ensure hydrolysis of such groupings. Completion of oxidation is indicated by the virtually steady state reached after several days.

Molecular chains terminated at the reducing end by mannitol units linked at C_6 would require an uptake of four moles at this position; on the assumption that 50% of laminarin molecules are thus terminated (50), the average periodate uptake at the reducing end would be 3.5 moles. Together with two moles from the non-reducing end, the total average periodate uptake per laminarin chain should thus be 5.5 moles.



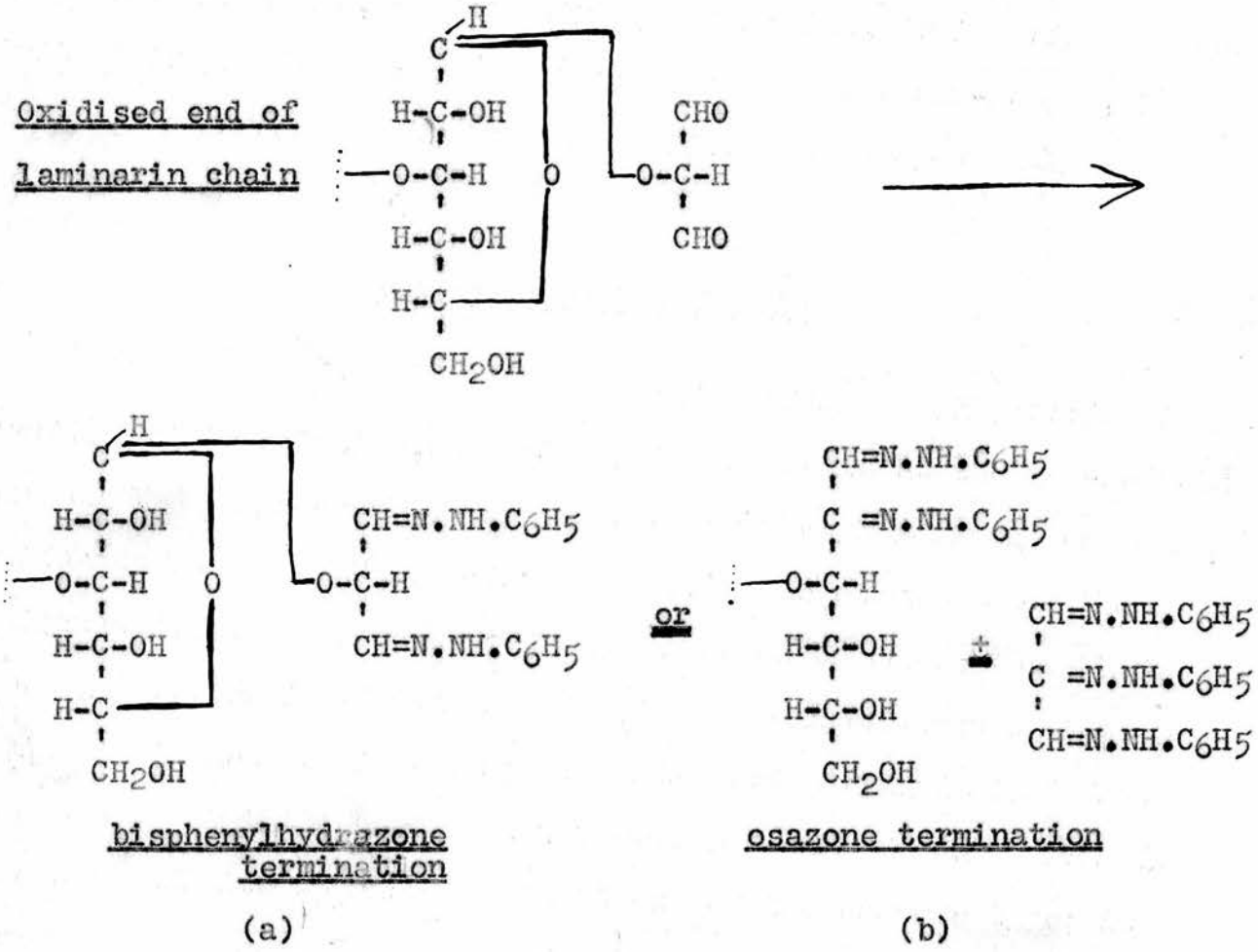
2 moles + 0 moles + 3 moles or 4 moles

On this assumption, the experimental value for periodate uptake, i.e. 1 mole per 710 g., or 5.5 moles per 3,900 g., would indicate an average chain-length of 24 sugar units. The amount of formic acid liberated by a molecule of the above structure should be 3.5 moles; 1 mole per 1230 g. (the experimental value, or 3.2 moles per 24 units, is in fair agreement with this prediction. The average value for chain-length, 24 units, is in agreement with that arrived at independently from other sources (50).

Degradation of Laminarin

On heating with phenylhydrazine in weakly acidic solution, the aldehydic fragments at the non-reducing end of the polysaccharide are removed as phenylhydrazone compounds of low molecular weight (cf. page 103). The other end of the polysaccharide would reasonably be expected to contain combined phenylhydrazine in either the phenylhydrazone or osazone form. While it is not possible at present to decide definitely between the two possibilities, the U.V. spectrum of the degraded material shows a definite similarity with those of the osazone type; it is quite different from that of methoxy-malondialdehyde bisphenylhydrazone (λ_{max} 243, 285 (51)), which it might reasonably be expected to resemble in the event of the reaction occurring as in (b) (cf. over). Similarly in the case of chains formerly terminated by mannitol residues, it is not possible to decide whether the chains after degradation are terminated by phenyl-

hydrazone groupings, or by osazone residues. However, general considerations on the ease of osazone formation, together with the absence of any indication of typical phenylhydrazone spectra, would appear to indicate that in this case also the molecule is terminated by osazone-like residues.



(a) (b)

If the osazone reaction does in fact take place, then degradation should leave an average of two combined phenylhydrazone residues per chain. The amount of nitrogen in the degraded polysaccharide (0.94%) leads to the calculation of 2.7 phenylhydrazone residues per 22 sugar sugar units, in fair agreement with the proposed reactions. It has

been suggested that at least part of the nitrogen in degraded polysaccharides is due to adsorbed phenylhydrazine or N-acetyl phenylhydrazine (52). Spectroscopic comparison of the degraded material with these substances precludes such a possibility in the present instance (cf. page 147).

Partial hydrolysis has led to the discovery of some 1:6-linkages in the laminarin molecule (53); so far it has not been definitely decided whether these linkages occur as branch-points in the polysaccharide, or as anomalous linkages in the 1:3-linked chain. In the latter case, unless the 1:6-linkages are present near the ends of the polysaccharide chain, degradation would be expected to yield a polysaccharide of low molecular weight, which would be of such a size as to pass through a dialysis membrane (cf. nigeran, page 102). The fact that at no stage was such a degraded polysaccharide encountered favours the occurrence of 1:6-linkages at branch-points in the molecule.

Further Oxidations and Degradations of Laminarin

Since degradation has of necessity left the non-reducing end of the molecule open to attack as in the original polysaccharide, periodate uptake at this position should be two moles as before. Interpretations of reactions at the other end of the polysaccharide are rendered uncertain by lack of knowledge concerning the effect of periodate on phenylhydrazone groupings. It has been well established that such groupings are

in fact attacked by periodate (cf. Part III, this thesis); no simple quantitative rule on the amount of uptake is apparent. If however the phenylhydrazone residues are present as the osazones of C_3-linked sugars, then it is at least possible to state that two moles of periodate will be consumed between C_{4-6} of the osazone unit. It can thus be assumed that at least four moles of periodate should be consumed by the degraded polysaccharide, together with an unspecified amount for the phenylhydrazone residues.

The experimental uptake for laminarin O_1D_1 was 1 mole per 480 g. of polysaccharide, i.e. 7.2 moles per 22 sugar units (the probable chain-length of the degraded polysaccharide). Apparently therefore three moles of periodate were consumed by the phenylhydrazone residues of the osazone molecule. In keeping with the theory of attack at this position, the U.V. spectrum of laminarin D_1O_2 exhibited no characteristic absorption peaks; furthermore the amount of nitrogen in the oxopolysaccharide was half that in laminarin O_1D_1 (0.51 and 0.94% respectively). Degradation with phenylhydrazine afforded laminarin O_2D_2 in 87% yield, and with nitrogen content similar (1.01%) to that of the once-degraded material. Again the degraded polysaccharide gave spectroscopic evidence of combined nitrogen nature, similar to that of laminarin O_1D_1.

Oxidation of laminarin O_2D_2 afforded periodate uptake of 1 mole per 610 g., i.e. 5.3 moles per 20 units (the probable chain-length of the twice-degraded material). The oxidised material had similar nitrogen content to that of

laminarin D_1O_2 (0.48 and 0.51% respectively), and again exhibited no absorption peaks in the osazone region. Since the amount of material available had by this time become very limited, no further reactions were carried out. For convenience, a tabulated summary of the experiments on laminarin is reproduced forthwith :-

	Periodate	Acid	%Yield	%N	Absorption max.
1st.Oxidation	1 mole/ 710 g.	1 mole/ 1230 g.	87	-	
Degradation	-	-	79	0.94	385
2nd Oxidation	1 mole/ 470 g.	1 mole/ 1230 g.	80	0.51	-
Degradation	-	-	87	1.01	375
3rd Oxidation	1 mole/ 610 g.	1 mole/ 1380 g.	76	0.48	-

Summarising, it can be concluded that the first oxidation of laminarin is in good quantitative agreement with data deduced elsewhere. Degradation affords a material with nitrogen content in fair agreement with the proposed picture of the molecule. A second oxidation of the polysaccharide gave periodate uptake of the order to be expected, although uncertainties as to the nature of reaction at the nitrogenous residues render precise calculation difficult in this case. A second degradation, followed by a third oxidation, afforded results similar to those already found. It is probable that the small amount of nitrogen (ca. 1%) in the degraded materials is present in the form of osazone groupings; the nitrogen content is reduced to half on re-oxidation.

It is evident therefore that the Barry degradation procedure is satisfactory in laying open to

periodate attack residues which had been previously shielded by other sugar units nearer to the periphery of the molecule. The yield of polysaccharide at each stage is of the order of 80%. While quantitative deductions from periodate uptake in the case of the degraded polysaccharides are subject to several unknown factors, the degree of oxidation is always of the expected order. It was with these conclusions in mind that attention was turned to the investigation of cladophoran.

PART II (b) - DISCUSSION

Oxidation and Degradation of Cladophoran

The polysaccharide extract from Cladophora rupestris affords on hydrolysis galactose, glucose, arabinose, xylose and rhamnose (39); methylation experiments have confirmed that the material is of very complex structure. The presence of ca. 20% of sulphate, together with lack of evidence as to the precise location of the sulphate grouping, has made it impossible to advance any general structure for the polysaccharide. It has previously been found that periodate oxidation destroys all the xylose units; it was with the intention of removing other sugar units, and so arriving at a simpler polysaccharide residue, that Barry degradation experiments were initiated on the extract. For convenience, a tabular summary of the results is reproduced:-

	Periodate (moles/1,000g.)	Sugars (rel.moles) Arab.	Gal.	Glu.	Xyl.	Rhm.	%Yield	SO4%	N%
Polysaccharide	-	4.0	3.0	0.2	1.1	0.5	-	18.3	-
1st Oxidation	2.82	4.0	0.9	0.3	-	0.7	89	19.2	
Degradation	-	"	"	"	-	"	67	16.6	3.5
2nd Oxidation	1.97	4.0	0.9	-	-	0.7	80	17.5	2.7
Degradation	-	"	"	-	-	"	66	16.9	4.2
3rd Oxidation	2.52	1.0	1.0	-	-	0.6	73	15.2	2.4
Degradation	-	"	"	-	-	"	74		
4th Oxidation	3.4	+	+	-	-	+			

First Oxidation and Degradation

The polysaccharide had periodate uptake of one mole per 360 g., corresponding to about one mole of oxidant per two sugar units. However since galactose probably present as end-group would be destroyed (39), and since each of these units would consume two moles, it is not permissible to assume that one mole consumed by every second sugar unit represents a true picture of the reaction. Isolation of the oxopolysaccharide confirmed the total destruction of the xylose residues, together with a large reduction (3 to 1 moles) in the amount of galactose. The occurrence in the hydrolysate of methylated cladophoran of 2:3-di-O-methylxylose, together with 2:3:4- and 2:3:5-tri-, and 2:3:4:6-tetra-O-methylgalactose, is in agreement with the changes in sugar content on oxidation. However, it is to be noted that the amount of highly-methylated galactose(ca. 35% of the total galactose content of the methylated hydrolysate) is not sufficient to explain the large relative destruction of this sugar on oxidation. The slight increase in sulphate (0.9%) in the oxopolysaccharide is probably due to the small decrease in weight of the polysaccharide on oxidation.

Degradation of the oxidised material afforded a polysaccharide O_1D_1 in 67% yield, reflecting the loss of a portion of the molecule through removal of aldehydic fragments. Chromatographic examination of a sample hydrolysate revealed that degradation had not affected the relative proportions of the residual sugars. The degraded polysaccharide had sulphate 16.5%, roughly comparable to that of the original material; it was thus

indicated that some of the ester groupings had been removed on degradation. Spectroscopic examination of the material confirmed that the nitrogen present (3.45%) was probably in the form of osazone groupings, as in the case of laminarin O_1D_1. The higher amount of nitrogen is probably a reflection of the greater degree of branching within the cladophoran molecule.

Investigation of the ethereal extract from the degradation reaction afforded glyoxal bis-phenylhydrazone; this substance can be traced to the oxo-product of 1:4-linked xylose, and also to the galactose units with free hydroxyl groups at C_2 and C_3 (cf. page 103). No other phenylhydrazone fragments were detected, although the presence of a large amount of N-acetyl phenylhydrazine may be responsible for failure to detect such substances as glycerosazone, which has very similar solubility and chromatographic properties,

Second Oxidation and Degradation of Cladophoran

Periodate uptake was equivalent to 1 mole per 510 g., i.e. per three sugar residues approximately. However, chromatographic examination of hydrolysed D_1O_2 revealed that the only change in the relative proportions of the different sugars was the disappearance of glucose (ca. 5% of the total molecule). It may be that uronic acid residues (5% of the original polysaccharide) have been laid open to attack by the first degradation, and that each of these has consumed two moles of periodate. However, it appears more probable that each of the other sugar residues has been attacked to approximately the same extent in this second oxidation.

The disappearance of glucose, which was verified by a second experimental oxidation, is difficult to understand. Earlier work on cladophoran had indicated that the glucose in the water-soluble extract was present as a 1:3-linked glucan of the laminarin type, and was immune to periodate attack except at the end of the chain. The possibility of loss by dialysis during removal of the inorganic material is not borne out by experience in the case of laminarin. The recent isolation of a sulphate-free glucan by aqueous extraction of mildly delignified Cladophora rupestris weed is of particular interest in this regard (54); the material gave a blue-green colour with iodine, and was totally destroyed by periodate. It may be that a trace of this polysaccharide is responsible for the glucose in the aqueous extract; if so, then it is so bound up with the rest of the material that an initial oxidation and degradation is necessary before the glucan is laid open to attack.

Cladophoran D_1O_2 had slightly increased sulphate content as compared with the once-degraded material; as in the case of the first oxidation, such increase is probably due to the removal of fragments of oxidised sugar. As was found with laminarin D_1O_2, the oxopolysaccharide gave no spectroscopic evidence for the presence of osazone groupings; the reduction in nitrogen content (3.45 to 2.7%) is not so marked as in the case of the simpler polysaccharide,

As before, degradation afforded a material with similar sulphate and increased nitrogen (4.2%) as compared to the oxopolysaccharide. No change was observed in the neutral sugar content.

Third Oxidation and Degradation

Cladophoran O_2D_2 had periodate uptake of 1 mole per 390 g. of polysaccharide, corresponding to an approximate consumption of one mole of oxidant per two sugar units. In keeping with this figure, it was found that the proportion of arabinose to galactose in the hydrolysate had changed from about 4:1 to 1:1, indicating destruction of ca. 75% of the arabinose residues, or ca. 50% of the total sugar content. Cladophoran D_2O_3 had similar sulphate content(15.2%) to that of the twice-degraded polysaccharide (16.9%); as in the previous instances, the nitrogen content (2.4%) was smaller than that of Cladophoran O_2D_2. Degradation afforded a material in which the same sugars were present as in the oxopolysaccharide.

Fourth Oxidation of Cladophoran

Periodate uptake of cladophoran O_3D_3 was still higher than that of the twice-degraded molecule, being equivalent to about one mole per 1.5 sugar units. The extensive attack indicated by these figures was further evident when it was found that cladophoran D_3O_4 was of such a size as to pass through the membrane of dialysis tubing, which had previously been used to remove inorganic material from the other stages of the polysaccharide. Hydrolysis of a sample showed that arabinose, galactose and rhamnose were still present; it is not clear how periodate can attack the ring-structure of a molecule causing fission of the polysaccharide chain. An explanation of this

effect must await further investigations on the polysaccharide.

Summarising then, application of the Barry degradation to cladophoran affords increasingly simple forms of the polysaccharide. Except possibly for the second degradation, amounts of periodate uptake are in agreement with the changes in sugar content. The nitrogen in the degraded polysaccharides is in the form of an osazone grouping, and is attacked on re-oxidation. One clear fact which emerges is that the sulphate ester groupings are spread relatively evenly over the surface of the molecule, and cannot be assigned to either peripheral or central sugars alone.

To obtain an unambiguous picture of the molecule, methylation at every oxidised and degraded stage would be desirable. In the present instance however, it was decided to methylate the thrice-oxidised polysaccharide; it was considered that the relatively simple proportions of sugars on hydrolysis at this stage indicated a less complex structure, which would afford a relatively simplified methylated hydrolysate.

Methylation of Cladophoran D_2O_3

Sulphated polysaccharides have always proved difficult to methylate fully; in the present instance the thallium procedure (55), which had proved of advantage for the Acrosiphonia extract, was found efficacious. The material finally isolated had methoxyl content of 26.9%, as compared with 25.1% for the original undegraded methylated material. The methylated hydrolysate was contaminated by

nitrogenous material from the phenylhydrazine treatment, but most of this material was easily removed as a fast-flowing mixture on the cellulose column used to fractionate the methylated sugars. In no case was a sugar with two contiguous methoxyl groups isolated, as was to be expected for a polysaccharide which had been subjected to periodate oxidation. The following sugars were separated and characterised :

2:4-di-O-methylrhamnose (1 part); 2:4-di-O-methylarabinose (4); 2:4:6-tri-O-methylgalactose (3) ; 6-O-methylgalactose (1); 2-O-methylarabinose (1); L-rhamnose (3); D-galactose (1).

With the exception of rhamnose, which is somewhat higher in the methylated hydrolysate, the relative proportions of these methylated derivatives is in good agreement with those of the sugars in cladophoran D_2O_3. It follows that these linkages are present at the centre of the molecule :

..3Rl..	..3Rl.. (2, 4)	..3Al..	..3Al.. (4)	..3Gal..	..3Gal.. (2, 4)	..3Gal.. (2, 4, 6)
(1)	(3)	(4)	(1)	(3)	(1)	(1)

The sulphate content of unmethylated D_2O_3 (15.2%) is equivalent to about one sugar residue in four or five carrying a sulphate grouping. It is not possible to allocate the residues to definite sugar units. The 1:3-linked galactose is unlikely to be present as end-group carrying sulphate on C_3, as such sulphate would be labile to the alkaline conditions of methylation and give rise to 3:6-anhydro-galactose (56). If sulphate groups are linked to galactose at all, then, since they are not labile to alkali, they are probably /

located at C_4; but only one in seven residues are galactose units with C_4 available for linkage. It appears then that at least some of the sulphate groups must be carried by either arabinose and/or rhamnose, with the large amount of free rhamnose indicating that this is the more likely unit; if linked to arabinose then the ester groupings are probably situated at C_3. A more definite answer to such questions must await the advent of other methods of investigation.

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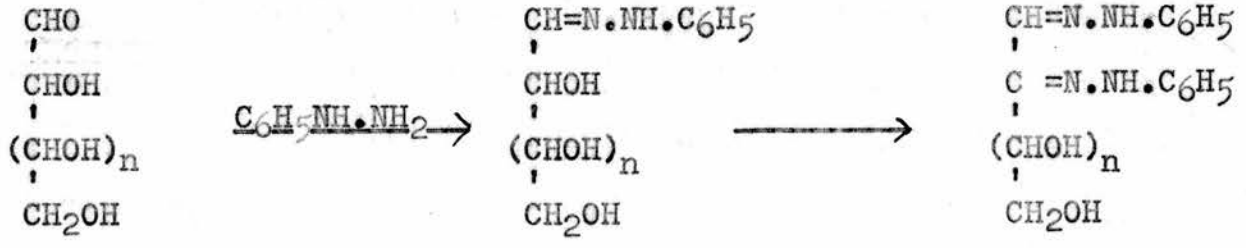
PART III

The Action of Periodate on Compounds Containing the Phenylhydrazone Grouping

The Action of Periodate on Compounds containing the Phenylhydrazone Grouping

In the course of the work described in the previous section it became apparent that the periodate ion attacked the phenylhydrazone residues in the degraded forms of laminarin and cladophoran. In each case the nitrogen content was reduced on oxidation, in laminarin to half the original value. In an attempt to elucidate the nature of this attack the work described in this section was carried out.

The potential aldehydic group on C_1 in reducing sugars has the property of condensing with the primary amino group in such compounds as hydroxylamine, aniline and phenylhydrazine. The last-named compound has found wide use in the preparation of crystalline mono- and di-saccharide derivatives which have proved of value in identification (1). Although the diphenylhydrazones (or osazones) (111) have been favoured for this purpose, it is with the monophenylhydrazones that this review is mainly concerned.



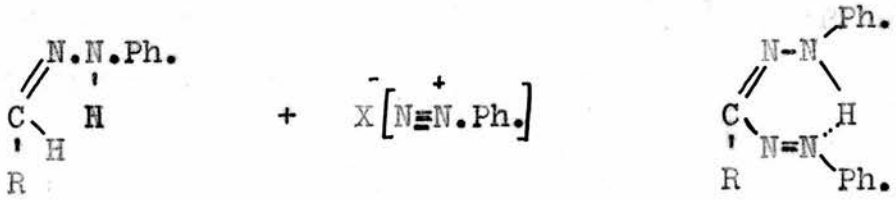
(11) (111)

Three distinct forms of glucose phenylhydrazone, with widely different melting point, but with

similar equilibrium rotational values have been isolated (2). In general solutions of sugar phenylhydrazones exhibit complex rotational changes which do not follow first order reaction rates, and it has therefore been assumed that more than one form of the hydrazone is present in solution (3). It was thus inferred that sugar phenylhydrazones exist in the ring form, as distinct from the open-chain form (11) in which they are usually written.

It has been shown that, in dilute acetic acid, glucose phenylhydrazone decomposes with concurrent formation of glucosazone (4). In view of this relative ease of decomposition in solution, it is doubtful whether the complex rotational changes observed can be explained solely in terms of mutarotation.

Information on the structure of the of the glucose phenylhydrazones has been obtained by application of the formazan reaction (5). This involves coupling the phenylhydrazone (1V) with diazotised aniline (V), resulting in the immediate formation of a red precipitate of formazan (V1). Since only acyclic phenylhydrazones may participate in this reaction, any precipitation on addition of diazotised aniline indicates an acyclic structure for the particular compound.



(1V) (V) (V1)

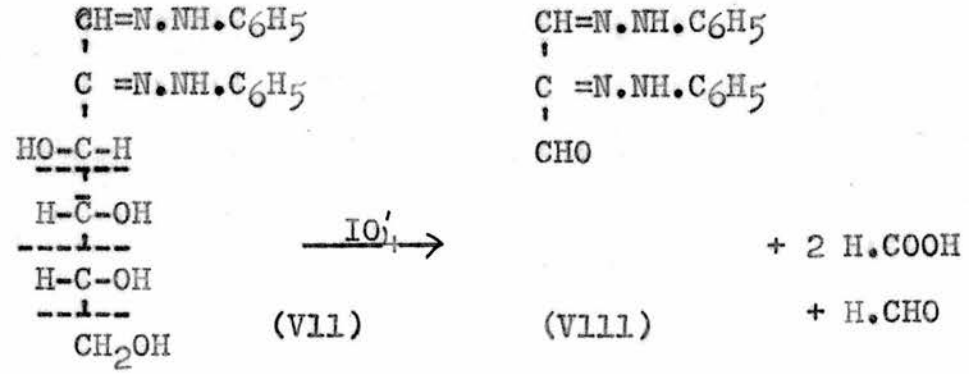
The various isomers of glucose gave

the following results :-	M.P.	Yield of Formazan
α-D-glucose phenylhydrazone	159°	0%
β-D-glucose phenylhydrazone	141°	67%
Skraup's phenylhydrazone	116°	0%

On the basis of this reaction it would appear that the α-form is cyclic in nature, together with Skraup's phenylhydrazone, while the β-form is acyclic. Mannose phenylhydrazone gives a high yield of the characteriatic red precipitate, indicating its acyclic nature.

It is reasonable to expect that compounds which contain a similar phenylhydrazone grouping would have this similarity exhibited in their absorption spectra in the chromophoric region; it is also possible that differences in cyclic structure would also thus be revealed. Such similarities have been correlated in the sugar osazone series (6), and it was partly to investigate any possible similar correlation in the phenylhydrazone series that the experiments described below were initiated.

It was early reported that glucosazone rapidly consumes three moles of periodate, with formation of insoluble mesoxaldehyde 1:2-bisphenylhydrazone (7) (V11) :-



It was noted (8) that this rapid theoretical uptake of periodate was followed by a slow increase in consumption which could only be attributed to interaction of the oxidant with the insoluble mesoxaldehyde bisphenylhydrazone. Reaction at different pH made no appreciable difference to the consumption, and it was observed that the iodate ion, IO_3^-, was also functioning as an oxidising agent. Quantitative measurements on glyoxal bisphenylhydrazone revealed that ca. 0.8 moles of periodate were consumed by the molecule within 24 hours. It has been similarly found elsewhere that with both N-acetylphenylhydrazine and 2:4-di-O-methyl-D-xylonic acid phenylhydrazide the uptake of periodate was approximately two moles per mole of nitrogenous material, again indicating oxidative attack at the n-atom (9). N-Phenylhydroxylamine consumes one mole of periodate, yielding nitroso-benzene, whereas the uptake in the case of aniline is at least three moles (10).

It seemed of interest to determine whether a similar reaction takes place with phenylhydrazine, and whether the ring-structure of the isomeric glucose phenylhydrazones might be evident in terms of periodate uptake. At the same time it appeared likely that additional information would be gained by investigation of periodate uptake of such substances as malondialdehyde tris-phenylhydrazone (11). It was considered possible that the uptake of such model compounds might be related to that of the degraded polysaccharides encountered in Part II of this thesis. To this end a number of simple phenylhydrazone compounds were prepared and oxidised.

EXPERIMENTAL

EXPERIMENTAL

Preparation of Monosaccharide Phenylhydrazones

In order to correlate the U.V. spectra of sugar phenylhydrazones, several monosaccharide derivatives were prepared. Since these compounds vary widely in solubility and ease of isolation, the preparation of each is described in detail.

Mannose Phenylhydrazone - Mannose (2 g.) was dissolved in 75% acetic acid (5 ml.) and the cooled solution left overnight with phenylhydrazine (1 ml.) at 0°C. The precipitated crystals were washed with ethanol and water till colourless, and then dried in vacuo (2.6 g., m.p. 188°.

Galactose Phenylhydrazone - Galactose (5 g.) was dissolved in 75% acetic acid (10 ml.), and phenylhydrazine (3 g.) added to the cooled solution. The mixture gradually solidified on shaking. After 16 hr. the solid mass was washed once with water, twice with a minimum of ethanol, and finally with ether until no trace of phenylhydrazine remained. The crude product (6 g.) had m.p.138°; successive recrystallisations from a minimum of absolute ethanol gave white crystals with m.p. 143°.

Glucose Phenylhydrazone (a) - Glucose (12 g.) was dissolved in 75% acetic acid at 100°; after cooling, phenylhydrazine (8 g. in 80 ml. of ethanol) was added to the stirred solution. A yellow-white precipitate appeared after one hour, precipitation being judged to be complete after 8 hours. The yellowish crude product

was then centrifuged off, and washed twice with a minimum of acetone. Prolonged washing with ether afforded a crude product with m.p. 110-112°. The material was dissolved in ethanol and reprecipitated with ether; repetition of the process afforded a white product with m.p. 116-117°, evidently the isomer known as Skraup's phenylhydrazone.

Glucose Phenylhydrazone (b) - A portion of the material described above was dissolved in a minimum of pyridine, and reprecipitated by addition of ether. Prolonged washing until the product was free from traces of pyridine, followed by drying in a vacuum desiccator, afforded the β-form of glucose phenylhydrazone as white crystals of m.p. 139-140°.

Glucose Phenylhydrazone (c) - Despite various attempts at isolation of the α-isomer by recrystallisation from hot absolute methanol or ethanol, in no case could a product with m.p. above 140° be isolated. A precipitate of the Skraup isomer, sometimes in admixture with the β-form, was invariably given. These difficulties were attributed to nucleation of the laboratory with the previously-prepared isomers, and further attempts to prepare the α-isomer were abandoned.

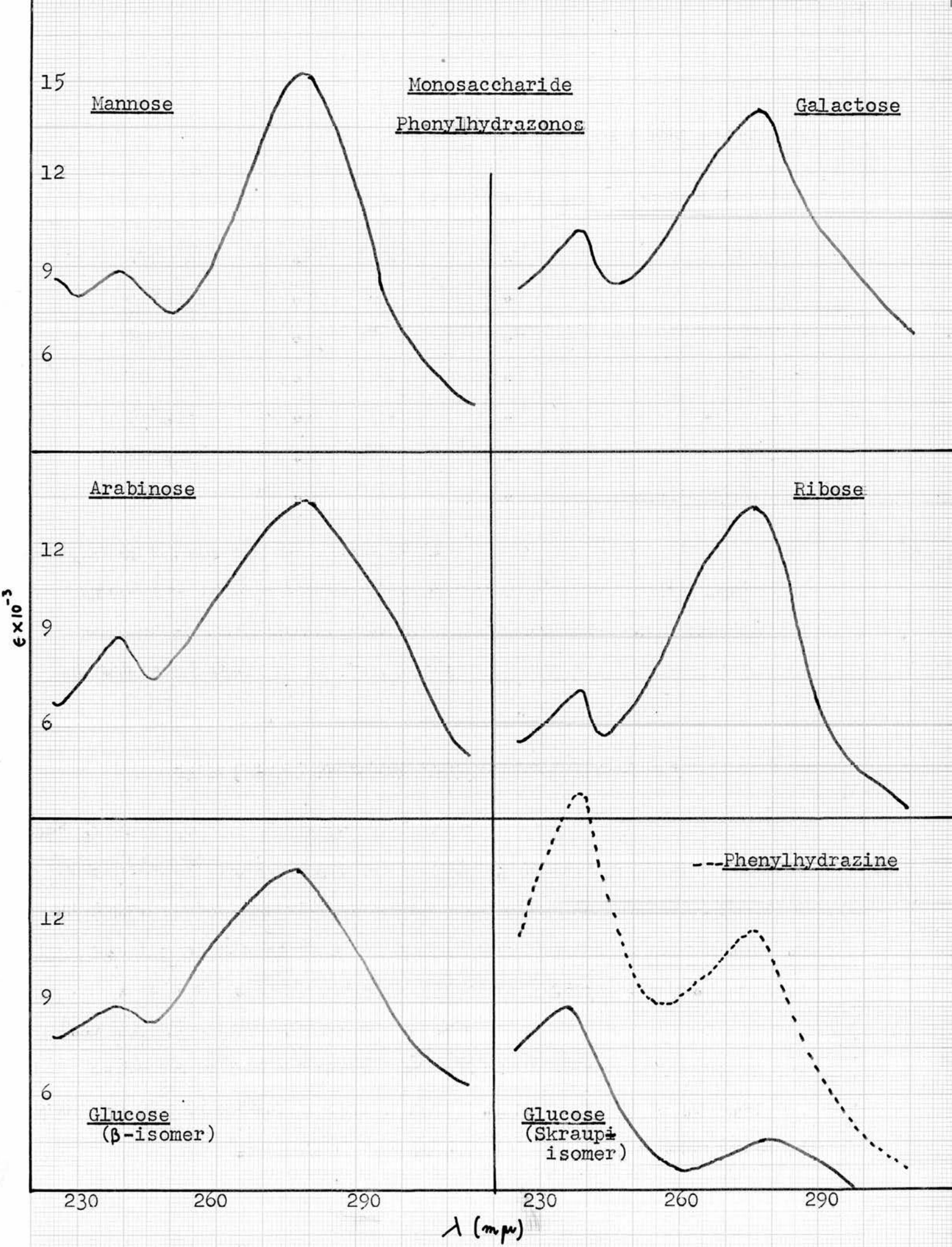
Arabinose Phenylhydrazone - Arabinose (8 g.) was dissolved in 75% acetic acid (10 ml.) and phenylhydrazine (5 ml.) was added to the cooled solution. On shaking over eight hours the mixture became solid. Water was then added, and the crude product centrifuged off and dried in vacuo. Recrystallisation from ether afforded white crystals of m.p. 141-142°.

Ribose Phenylhydrazone - Ribose (5 g.) was treated with acetic acid and phenylhydrazine as in the case of arabinose, affording a solidified yellow mass on shaking. After eight hours water was added and the mixture exhaustively extracted with ether. Evaporation to dryness, followed by repeated washing with benzene, afforded a pale-yellow product largely free from phenylhydrazine and osazone (5 g.). Recrystallisation from a minimum of ether afforded white crystals with m.p. 112°.

Xylose Phenylhydrazone - Many attempts were made to prepare xylose phenylhydrazone, including the procedures above and modifications at 0° with varying amounts of acetic acid. In all cases the product was yellow crystals of m.p. 138°, similar to those described in the literature (12). Since however this material gave a characteristic osazone spectrum, further experiments on the derivative were abandoned.

U.V. Spectra of the Various Phenylhydrazones

Solutions of each of the derivatives were made up in 50% ethanol at a concentration of ca. 10 mg. per litre; the absorption spectrum of each freshly-prepared solution was then measured in the range 220 - 320 mμ. Except in the case of the Skraup isomer, all the phenylhydrazones were characterised by an absorption band of maximum intensity ca. 278 mμ. Phenylhydrazine exhibits a similar absorption band in the same region. A comparison between the various molecular absorption coefficients is afforded in the /


Monosaccharide
Phenylhydrazones
Mannose
Galactose
Arabinose
Ribose
Glucose
(β-isomer)
Glucose
(Skraup
isomer)
---Phenylhydrazine
15
12
9
6
ε×10⁻³
230
260
290
λ (mμ)

following table :-

Phenylhydrazone	c. (mg./litre)	mμ	E	ε
Mannose	9.68	278	0.545	15,100
Galactose	9.68	278	0.492	13,800
Glucose (β-form)	9.68	278	0.503	14,200
Glucose (Skraup isomer)	9.68	237	0.320	8,900
Arabinose	8.57	276	0.480	13,300
Ribose	8.57	278	0.482	13,400
(Phenylhydrazine)	4.32	275	0.454	11,400

In the case of the second absorption peak (ca. 237 - 240 mμ), no regularity of absorption intensity is discernible. The Skraup isomer of glucose phenylhydrazone is anomalous in that the absorption peak of highest intensity is at the lowerewave-length.

Periodate Oxidation of the Phenylhydrazones

A typical oxidation is described, the procedure being essentially similar in each acse. Measurements of oxidant uptake were carried out by the Fleury-Lange method on 0.5 ml. samples; acidity was estimated by titration to pH 6.2 against 0.0284 N sodium hydroxide solution (2 ml. samples).

Mannose phenylhydrazone (152.5 mg.) was suspended in water (7 ml.), and sodium periodate solution (21 ml., 0.197 M) was added. The course of reaction was followed by withdrawal of samples at the stated intervals.

Blanks:

4 ml. of arsenite soln(0.05 N) = 22.4 ml. of iodine soln. (0.0089 N)
4 " " " " + 0.25 ml. of periodate soln(0.197M)=5.7ml. " " " .

Time (hr.)	0.1	0.25	0.5	1	2	8	24	48
I_2(0.0089N,ml)	13.2	14.5	14.6	15.7	17.7	18.0	19.1	20.6
NaOH(0.0284N,ml)			4.1				5.5	

Periodate Uptake : After 30 min. :- 4.01 moles
" 48 hr. :- 6.75 " .

Acid Produced : After 30 min. :- 2.88 moles
24 hr. :- 3.95 " .

After 30 min. of oxidation, an aliquot (10 ml.) was removed from the solution and treated with ethylene glycol and sodium bicarbonate. The red crystalline material which had separated during oxidation was removed by centrifugation, washed sparingly with water, and dried in vacuo (20 mg., 80%). The crude product melted ca. 75°; recrystallisation from ethanol-water afforded red crystals of m.p. 85°. The recorded melting-point of glyoxal monophenylhydrazone is 89° (13). Analysis of the material gave :- C 63.6; H 6.4; N, 18.4%. Calc. for $C_8H_8N_2O_1$:- C, 64.8; H, 5.4; N, 18.9%.

Oxidation of other Phenylhydrazones

The other phenylhydrazones were treated with periodate as in the case of mannose phenylhydrazone, the results being summarised in the following table :

Phenylhydrazone	Periodate Uptake(moles)		Acid Produced (moles)	
	30 min.	72 hr.	30 min.	72 hr.
Galactose	4.05	6.30	2.9	3.8
Glucose (β-isomer)	3.90	6.20	2.9	3.9
Glucose (Skraup-isomer)	3.95	6.80	2.7	3.8
Arabinose	3.06	4.60	2.0	2.6
Ribose	3.16	4.90	2.1	2.6

In all cases oxidation was accompanied by precipitation from solution of the characteristic orange crystals within the first hour. Aliquots were isolated in each case, and the similarity of the crystalline products confirmed by comparison of their U.V. spectra. As oxidation proceeded in the remainder of the solution, the precipitated crystals gradually redissolved, affording a brown tarry product. On one occasion this material was extracted from the oxidation mixture by dissolution in ether. The brown product resulting from evaporation had no distinguishing features in its U.V. spectrum.

Oxidation of other Phenylhydrazone Compounds

Several simple pehnylhydrazone compounds were prepared and their periodate consumption estimated as in the case of the sugar derivatives. The results are summarised in the following table :

	Time (hr.)		
	0.5	24	48
	Moles of Periodate per Mole		
Glyoxal Monophenylhydrazone	1.0	2.2	2.6
Glyoxal Bisphzone.(14)	0.6	0.9	1.2
Mesoxaldehyde Bisphzone.(7)	0.7	1.0	1.1
Malondialdehyde Trisphzone. (11)	0.8	1.8	2.2
N-Acetyl phenylhydrazine (15)	0.6	1.8	2.1
Phenylhydrazine	1.3	3.2	3.3

It was thus demonstrated that periodate attacks the phenylhydrazone grouping, no relation between the various uptakes being immediately apparent.

DISCUSSION

DISCUSSION

Preparation and Properties of Sugar Phenylhydrazones

All sugar phenylhydrazones were prepared by reaction with phenylhydrazine in acetic acid solution at normal temperatures over periods of eight to sixteen hours. In each case it was necessary to purify the product from contaminating phenylhydrazine and osazone; ease of osazone formation under the conditions employed, as indicated by the degree of yellowness of the reaction mixture, varied greatly from sugar to sugar. The extreme example was observed in the case of xylose, where no phenylhydrazone could be isolated. It is probable that the yellow crystals described as xylose phenylhydrazone are really xylosazone (12), since the product prepared as described had a characteristic osazone spectrum. With the notable exception of the mannose derivative, all the derivatives tended to decompose over several months, with the liberation of free phenylhydrazine; the pentose compounds were especially susceptible in this regard. It is probable that this decomposition bears relation to the observation that phenylhydrazones in weakly acidic solution are decomposed with concurrent formation of osazone (4).

The phenylhydrazones described exhibited wide difference in solubility, necessitating different methods of isolation. Solubilities ranged from that of the mannose derivative, almost completely insoluble in water or ethanol, to that of ribose phenylhydrazone, which was readily soluble in ether. Preparation of glucose phenylhydrazone under

the conditions used for the other derivatives afforded the Skraup isomer of m.p. 116°. When this substance was precipitated from pyridine by addition of ether, the β-isomer, of m.p. 139°, was obtained. All attempts to prepare the α-isomer afforded only one or both of the previously-isolated materials, this occurrence being attributed to nucleation of the laboratory by these substances.

With the exception of the Skraup-isomer of glucose phenylhydrazone, all derivatives exhibited similar spectra in the region mμ230-300. Each exhibited absorption maxima at mμ277-80, together with a second less well-defined peak at mμ235-40. Phenylhydrazine exhibits two absorption maxima at these wave-lengths; together with the Skraup isomer, maximum absorption occurs at the shorter wave-length in this case. The mean molecular absorption of four of the phenylhydrazones at mμ. 276-8 was 13,700, each of the individual values being within 4% of this value. The molecular absorption for mannose phenyl-hydrazone was 10% above this figure; similarly the value for free phenylhydrazine was 20% below the mean.

The anomalous nature of the Skraup isomer of glucose phenylhydrazone is in keeping with the formazan investigations discussed in the introduction; it was concluded that the β-isomer of glucose phenylhydrazone is acyclic in nature and similar to that of mannose; while the Skraup isomer was cyclic and essentially different to the β- and mannose phenylhydrazones.

Oxidation with sodium periodate has hitherto been applied to the sugar osazones, resulting in elimination of the glycol groupings, and with production of

insoluble mesoxaldehyde 1:2 bisphenylhydrazone (7). In the case of the phenylhydrazones a similar reaction takes place, although isolation of the resultant glyoxal monophenylhydrazone is rendered more difficult by the comparatively high solubility and relative ease of polymerisation of this substance. In all cases the theoretical uptake of periodate (from a glycol view-point) was complete within thirty minutes; oxidation was accompanied by production of the characteristic orange-red crystals of the monophenylhydrazone. The fact that analysis revealed the presence of one more H-atom than can be accounted for on the simple formula is noteworthy, and would indicate that some form of cyclisation not represented by the simple formula has taken place. The red crystals of monophenylhydrazone gradually redissolved on further oxidation, together with the formation of a brown tarry substance; as in the case of laminarin D_1O_2 and D_2O_3, the product of prolonged oxidation had no characteristic absorption spectrum. Since no correlation could be observed between the various uptakes of periodate at this point, it was decided to isolate glyoxal monophenylhydrazone afresh, and commence oxidation with the unnattacked substance.

The similarity of results in the case of the Skraup isomer are somewhat surprising, and would appear to indicate that periodate oxidation is not likely to prove of value in the elucidation of such structures; similar conclusions have been reached elsewhere in the case of allied sugar compounds (16)

Periodate Oxidation of Phenylhydrazones of Low Molecular Weight

In order to investigate more fully

the amount of periodate uptake by phenylhydrazone residues, several other simple phenylhydrazone compounds were prepared and oxidised. In each case a relatively rapid uptake of about one mole was followed by a further slow increase in periodate consumption; in the case of the bisphenylhydrazones of glyoxal and mesoxaldehyde the total uptake of periodate was of the order of one mole after 48 hours. The uptake of 0.9 moles by the glyoxal derivative is in agreement with the figure previously found by Courtois and Le Dizet (8); these workers found a value of 0.74 moles under slightly different conditions. The value for Nacetyl phenylhydrazine is in agreement with the results reported hitherto, wherein a value of 2 moles per mole was arrived at. The uptake of at least three moles by free phenylhydrazine is in harmony with similar results in the case of aniline (10).

Summarising, it can be said that phenylhydrazone compounds are attacked by periodate with the uptake of at least 1 mole between each bisphenylhydrazone residue. No apparent correlation exists between the total uptake in each individual case; furter investigation of this problem must await examination of a much wider range of compounds.

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ACKNOWLEDGEMENT

The author wishes to express his appreciation of the interest taken in, and the advice given by, Professor E.L.Hirst, F.R.S., during the course of this work. His most grateful thanks are also due to Dr. E.E.Percival, under whose kindly supervision and enthusiastic guidance the experimental work was carried out.

Our gratitude is also due to Dr. Beevers for helpful instruction in the carrying out of X-ray analyses, and likewise to Dr. Anderson for the determination of Infra-Red spectra.

In conclusion, we are indebted to the Institute of Seaweed Research, whose financial assistance made possible this research programme.

REPRINTED FROM CHEMISTRY AND INDUSTRY, 1958, p. 834

BARRY DEGRADATION OF LAMINARIN

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Recent work on insoluble laminarin has revealed the presence of some β-1:6-glucosidic linkages.[1,2] The molecule therefore may consist of unbranched chains of β-1:3-linked glucose units occasionally interrupted by β-1:6-links or it may be branched. In the absence of positive evidence for branching some workers[1] prefer to leave the question open. By contrast others[2] are of the opinion that the molecule is slightly branched with β-1:3-linked chains interlinked by β-1:6-glucosidic linkages, since 80% of the molecules (by weight) have a degree of polymerization of 60 and an average chain length of 23. Subjection of laminarin to successive oxidation with sodium metaperiodate and degradation with phenylhydrazine[3] gives, after three oxidations and two degradations a residual oxopolysaccharide (D_2O_3), the molecules of which are sufficiently large to be retained inside a dialysis sac, and which retain the characteristic property, of insoluble laminarin, of slow precipitation from aqueous solution.

The oxopolysaccharide is isolated at each stage by precipitation with alcohol after dialysis against running water (3 days). Degradation with phenylhydrazine is followed by exhaustive extraction with ether and isolation of the degraded polysaccharide (OD) by freeze-drying the aqueous solution. The yield and nitrogen contents of the materials isolated are given in the Table.

Table

	Weight (g.)	*Yield* (%)	*N content* (%)
Laminarin	4·16		
Oxopolysaccharide (O_1)	3·62	87	
Degraded oxopolysaccharide (O_1D_1)	2·86	79	0·94
Degraded oxopolysaccharide (D_1O_2)	2.29	80	0·51
Degraded oxopolysaccharide (O_2D_2)	2·00	87	1·01
Degraded oxopolysaccharide (D_2O_3)	1·52	76	0·48

The small loss observed during dialysis of O_1 is in accord with the postulated heterogeneity of the laminarin sample.

Periodate oxidation and phenylhydrazine degradation only attacks and removes the residues at either end of a 1:3-linked chain, but wherever 1:6-links occur the molecule is cleaved. Their presence at intervals along an unbranched molecule of average chain length of 23 would, under this treatment, give rise to small fragments of dialysable size. However, if the 1:6-links occur only as inter-chain linkages then chains of approximately the same length would remain after oxidation and degradation. Unless therefore the 1:6-links occur exclusively near the ends of the chains, the results now recorded support the idea of a branched structure for the laminarin molecule.

Received April 30, 1958

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